CEREAL CHEMISTRY

Published bi-monthly by

The American Association of Cereal Chemists

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Dept. of Cereal Technology, North Dakota State University, Fargo
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Second-class postage paid at Minneapolis, Minn. Published at 500 S. 5th St., Minneapolis, Minn. Subscription rates $18.00 per year. Foreign postage, $1.00 extra. Single copies, $3.25; foreign, $3.45. Back issues, $3.50. Claims for missing numbers will not be allowed if received more than 60 days from date of mailing plus time normally required for postal delivery of journal and claim. No claims allowed when subscriber has failed to notify the Circulation Department of a change of address, or because copy is missing from subscriber's files.

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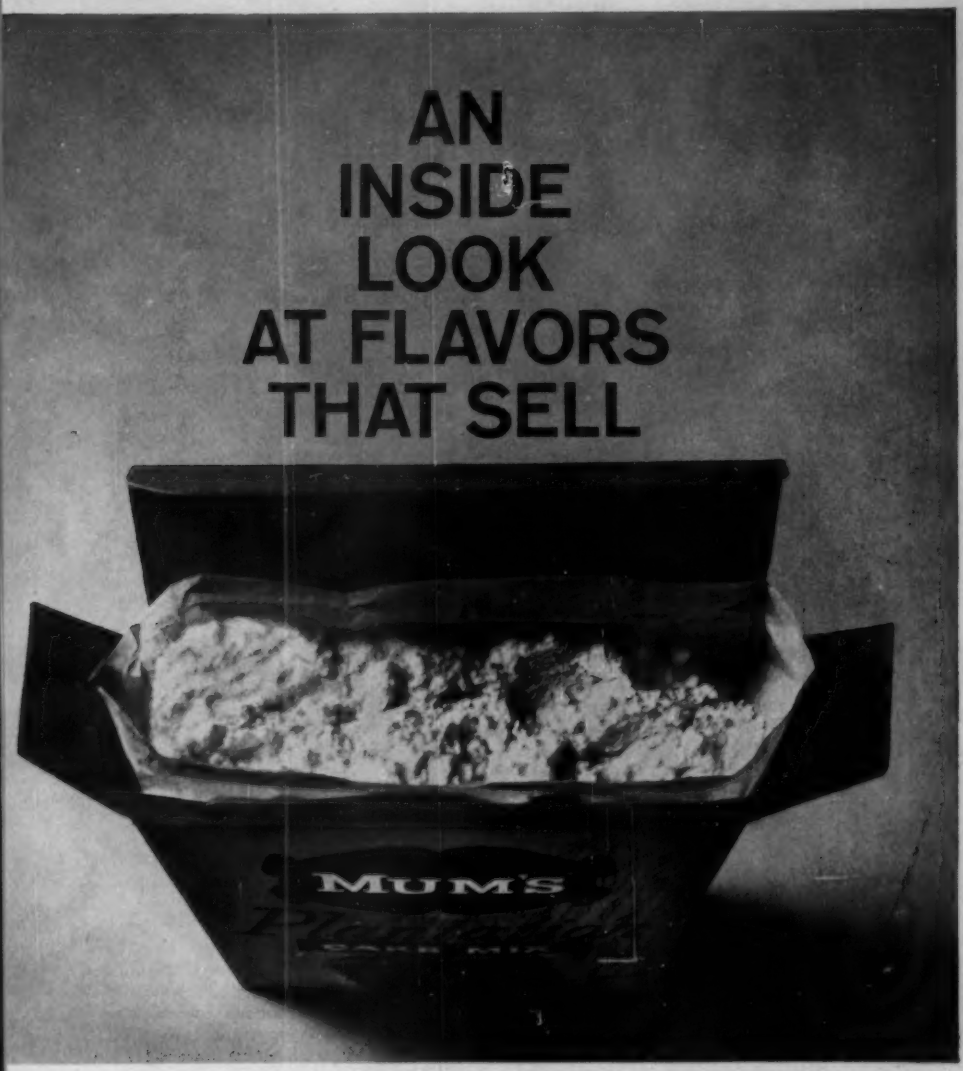

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CEREAL CHEMISTRY

VOL. 38 SEPTEMBER, 1961 NO. 5

SOME IMPROVING EFFECTS OF HALOGENATES AND THEIR REDUCTION INTERMEDIATES IN DOUGH[1]

R. TKACHUK AND I. HLYNKA

ABSTRACT

As the reduction of bromate in dough probably involves the intermediates bromite, hypobromite, and bromine, a comparison of the effect of hypobromite, bromine, chlorite, hypochlorite, and chlorine with bromate and iodate was carried out by extensigraph and baking studies on wheat doughs. The results indicate that the relatively slow reaction of bromate is due to the first reduction stage of bromate to bromite, and the first and second reduction stages (bromate → bromite →) cause the most efficient chemical reaction of the functional groups directly responsible for the improving effect in dough. Sodium chlorite compares favorably with bromate and iodate as a flour improver. The remaining reagents act as less efficient improvers.

In acidic aqueous solutions containing bromide, bromate is converted to bromine, and the following sequence of reactions has been postulated by Hinshelwood (11):

$$HBr + HBrO_3 \rightarrow \quad HBrO_2 + HOBr \quad \text{(Rate-controlling)}$$
$$HBr + HBrO_2 \rightarrow 2\ HOBr \quad \text{(Rapid)}$$
$$HBr + HOBr \rightarrow \quad Br_2 + H_2O \quad \text{(Rapid)}$$

Bromine in the presence of suitable substances such as oxalate, is reduced to bromide (11):

$$Br_2 \rightarrow Br^- + Br^+$$
$$C_2O_4^= + Br^+ \rightarrow 2\ CO_2 + Br^-$$

This hypothesis is, in general, consistent with the results obtained from other studies of the reduction of bromate (1,2,10).

It would seem reasonable then, to assume that the reduction of bromate (3,13) or iodate (5) in dough proceeds in a similar manner and involves intermediate reduction entities such as bromite, hypobromite, and bromine. Thus the improver effect of bromate may be partly due

[1]Manuscript received January 26, 1961. Paper No. 202 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg 2, Manitoba. Presented in part at the 45th annual meeting, Chicago, Illinois, May 1960.

to one of these intermediates or to a combination of all or most of them. Some aspects of this matter are examined in the present paper.

As it is difficult to prepare bromite free from mercuric salts (4), experiments were restricted to studies of hypobromite and bromine; but the corresponding chlorine homologues (10), chlorite, hypochlorite, and chlorine, were included together with the well-known improvers bromate and iodate. The effects of these reagents were tested by means of both extensigraph and baking methods. Gassing power was checked in order to ensure that baking performance was not affected by possible inhibition of the yeast.

Materials and Methods

Hypobromous acid was prepared by the addition of excess silver orthophosphate to an aqueous bromine solution. After the bulk of the insoluble material was removed by centrifuging, vacuum distillation yielded pure hyprobromous acid (1). Hypochlorous acid was prepared in a similar manner.

Hypobromous acid and bromine solution were freshly prepared every day; sodium chlorite, hypochlorous acid, and chlorine solutions every second day; and all were stored in amber-colored bottles at 4°C., with minimum exposure to light. All solutions were analyzed for their oxidizing power towards acidic potassium iodide solutions by amperometric titrations (6).

The flour was a commercial, straight-grade, unbleached and nonimproved sample (13.5% protein) from a blend of Canadian hard red spring wheat. Doughs for extensigraph studies were mixed under an atmosphere of nitrogen, and relaxation constants were determined as previously described (7,8). Baking tests were made by the basic AACC method with 2% yeast and an absorption of 59%; and gassing powers were determined by the AACC pressuremeter method (12).

Results and Discussions

The extensigraph data are given in Fig. 1 as a plot of the relaxation constant against the reaction time. Curves for the bromine and chlorine compounds tested are shown in the upper and lower portions respectively, as solid lines; for purposes of comparison, curves for bromate and iodate, and control doughs, are included as dotted lines.

Figure 1 shows that hypobromite is a more effective improver than bromine, but that neither is as effective as bromate. Moreover, whereas bromate reacts slowly, hypobromite and bromine react quite quickly in dough. This was confirmed by amperometric titrations of dough extracts, which showed that these two reagents were not present 4

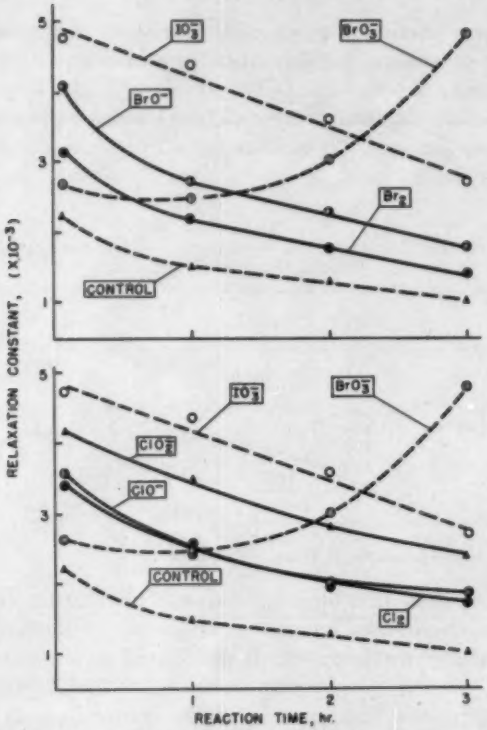

Fig. 1. Relation between the relaxation constant and reaction time. The dosages for Br_2, BrO^-, BrO_3^-, Cl_2, ClO^-, ClO_2^-, and IO_3^- are 125, 125, 75, 125, 125, 30, and 30 μeq. per 100 g. flour.

to 5 minutes after their incorporation into dough.

The lower portion of Fig. 1 shows the curves for doughs treated with chlorite, hypochlorite, and chlorine. All three act as improvers, and react rapidly as was confirmed by amperometric titration. Sodium chlorite is more effective than any of the other derivatives, but less effective than iodate or bromate; its effect on extensigraph properties of dough at a reaction time of 10 minutes has been noted previously (14). Hypochlorite and chlorine behave similarly, and are about intermediate in effectiveness between hypobromite and bromine.

It would have been ideal to have a direct comparison of all the reagents tested at a dosage corresponding to an equivalent oxidizing power. However, this is difficult to accomplish, because the range of the instrument is limited in that it cannot directly measure the large variation in dough properties caused by these reagents. Never-

theless, a very useful order of effectiveness of the reagents can be obtained by comparing the relaxation constants in Table I representing the dosages actually used. To obviate the difficulty of different dosages, calculated constants are also listed; these represent equivalent dosages of 30 μeq. per 100 g. flour for all reagents (i.e. for bromate, $4.8 \times 10^{-3} \times 30/75$).

TABLE I
RELAXATION CONSTANTS FOR NONFERMENTING DOUGHS CONTAINING VARIOUS HALOGEN COMPOUNDS

REAGENT	DOSAGE [a]	RELAXATION CONSTANT $\times 10^{-3}$	CALCULATED RELAXATION CONSTANT [b] $\times 10^{-3}$
BrO_3^-	75	4.8	1.9
IO_3^-	30	2.8	2.8
ClO_2^-	30	2.3	2.3
BrO^-	125	1.8	0.43
ClO^-	125	1.8	0.43
Cl_2	125	1.7	0.41
Br_2	125	1.4	0.34
Control	...	1.0	...

[a] Microeq. per 100 g. flour.
[b] Calculated to a dosage corresponding to 30 μeq. per 100 g. flour.

These data show that bromate, iodate, and chlorite are of the same order of effectiveness for increasing the resistance of dough to stretching, and that the remaining compounds tested are only about one-fifth as effective.

Baking tests were also made with the compounds. It was noticed that, with some reagents, the effect on loaf volume depended on the manner of addition and incorporation into the dough. For example, when hypobromite and yeast solutions were added separately to the flour just prior to mixing, only very slight loaf volume increases were obtained, even at dosages up to 344 μeq. per 100 g. flour. However, if the hypobromite and yeast solutions are added separately and simultaneously to a flour being continuously mixed, then reproducible loaf volumes resulted. The second procedure was therefore used in all the baking tests reported in this paper. Studies with each reagent also showed that, with this procedure, none had any effect on gassing power at dosages producing optimum loaf volumes – or at twice these dosages.

Loaf volumes obtained at various concentrations of each reagent are given in Fig. 2. Because one has to consider both the optimum loaf volume obtained and the dosage of the reagent used at this optimum (Table II), it is more difficult to list a definite order of efficiency. Nevertheless, it is obvious that there is a clear distinction between the behavior of bromate, iodate, and chlorite and of hypochlorite, chlorine,

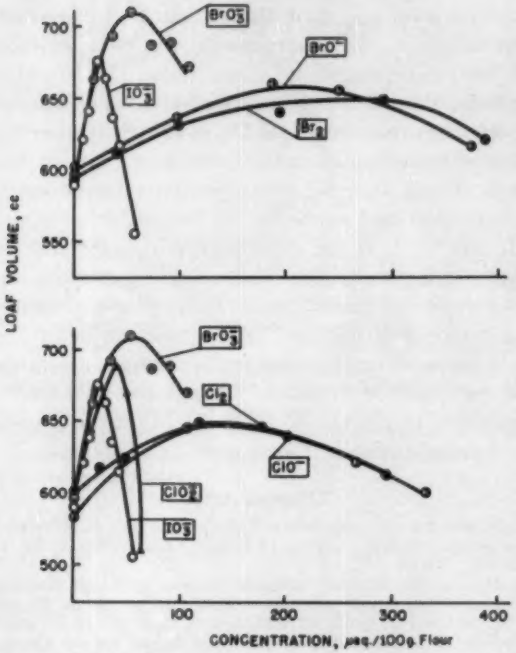

Fig. 2. Relation of loaf volume changes to added increments of various halogen compounds.

hypobromite, and bromine; the former group are at least twice as efficient as improvers. Thus we see that all the compounds tested increase the loaf volumes of baked bread, and in general these results are similar to and complement the results obtained from the rheological experiments. It is of interest to note here that chlorite produced

TABLE II
DOSAGES OF HALOGEN COMPOUNDS AT OPTIMUM LOAF VOLUMES

REAGENT	OPTIMUM LOAF VOLUME	DOSAGE AT OPTIMUM LOAF VOLUME	RELATIVE DOSAGE AT OPTIMUM LOAF VOLUME (21 μEQ. ≡ 1)
	cc	*μeq*	
IO_3^-	665	21	1
ClO_2^-	665	38	1.8
BrO_3^-	705	54	2.6
ClO^-	650	114	5.4
Cl_2	650	135	6.4
BrO^-	665	186	8.8
Br_2	640	200	9.5
Control	595	...	...

an optimum loaf volume slightly higher than that produced by iodate.

The first reaction in Hinshelwood's proposed series (11), given earlier, has been experimentally substantiated (1,2,10). Moreover, the present findings that bromine, hypobromite, and also bromite (by analogy to chlorite) react very quickly in dough further indicate that the reduction of bromate to bromite controls the rate of disappearance of bromate in dough also. All other reactions, including those of the chlorine homologues and probably the iodine homologues, appear to be rapid. It may be that the more highly oxidized compounds react with sulfhydryl groups, and that the remaining compounds are indiscriminate oxidizing reagents. Since it has been shown that small amounts of cysteinesulfenic and cysteinesulfonic acids are produced from cysteine by bromate (9), some indiscriminate oxidation of a similar type may be expected in dough. Though the principal reactions of bromate and other improvers in dough might yet prove to be relatively simple, the detailed stoichiometry is doubtless complex.

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PREPARATION OF PURIFIED ZEIN BY ADSORPTION-DESORPTION[1]

E. M. CRAINE,[2] DIANE V. FREIMUTH, JOYCE A. BOUNDY, AND R. J. DIMLER

ABSTRACT

A procedure was developed for preparing colorless zein that retains good solubility in aqueous ethanol. Zein was preferentially adsorbed from 70 volume-percent ethanol on a column of weak cation exchanger resin in the acid form and also was readily desorbed by salt in 70% ethanol. Isolation of the zein by slow precipitation from the aqueous ethanol through dialysis at 0° to 4°C. against water, followed by lyophilization, resulted in retention of good alcohol solubility. The nonhygroscopic, easily powdered product gives clear solutions in alcohol and merits consideration not only as a reference sample but also as a constituent of rations for animal feeding studies using zein. The purification procedure was shown to be applicable to unmodified zein extracted directly from corn meal and to commercial zein produced from gluten obtained in corn wet-milling.

Our need for reference samples in studies of the isolation and characterization of corn proteins led to a study of purification of zein. Two objectives were: the removal of nonprotein contaminants, including pigments, and the recovery of purified zein in a dry form retaining good solubility characteristics.

The removal of nonprotein impurities from zein generally has been accomplished by methods similar to those used by Chittenden and Osborne (1), who used ethanol and ether for extraction and precipitation. Removal of yellow pigments from zein by these solvents generally is incomplete. Extraction of pigments and lipids with ethylene dichloride was accomplished by Mason and Palmer (6); however, they gave no data on solubility characteristics of the zein which they prepared from commercial yellow corn gluten meal.

Incomplete solubility of zein preparations in aqueous ethanol has been noted in numerous reports on the purification and isolation of zein. In some cases insolubility may result from use of high temperature (1), repeated contact with different solvents, or a large number of extraction steps. Another important cause of low solubility appears to be the physical form in which the product is obtained. Precipitation of zein from an alcoholic solvent by addition of water or an organic solvent often yields a taffylike mass. Such a precipitate becomes horny

[1] Manuscript received November 7, 1960. Contribution from the Northern Regional Research Laboratory, Peoria, Illinois. This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

[2] Present address: The Upjohn Co., Kalamazoo, Mich.

on drying and requires mechanical grinding to reduce it to a powder. While small particle size after grinding improves solubility, such products often do not go into solution as readily as if the horny state were avoided. Some precipitation techniques that have been reported to give readily soluble zein, such as dispersing an alcohol solution of zein in cold water (4), did not give consistent results in our studies.

Some disadvantages of previous laboratory procedures for purifying and preparing dried samples of zein have been overcome in the present studies. A preferential adsorption of zein from alcoholic solutions by weakly acidic cationic exchange materials was observed. On this basis a method was developed for obtaining a highly purified white zein that gave clear, colorless solutions in ethanol. Retention of good alcohol solubility was achieved by modifying the procedure for precipitating and drying the zein.

Commercial zein and unmodified zein, which was extracted directly from ground corn, were included in these studies because of possible differences in their behavior. Commercial zein is prepared from corn gluten meal, obtained in wet-milling corn for starch. The sulfur dioxide used in the wet-milling process probably causes some chemical modification of the protein at disulfide (cystine) linkages. In addition, commercial corn gluten reportedly contains at least two types of zein protein, only one of which (alpha-zein) is extracted in the commercial preparation of zein (7,10).

Experimental

Materials and Analytical Methods. Commercial zein was supplied by the Corn Products Co.[3] as Lot No. HV-9 and had a nitrogen content of 14.7% (dry basis). This product may differ somewhat from current lots of commercial zein because of changes in processes.

Unmodified zein was prepared from whole ground yellow corn (Dyar 444 of the 1957 crop) which had been partially defatted with petroleum ether (2), dried, and then extracted with 70% (by volume) ethanol at 28°C. The extract contained about 35% of the total corn nitrogen. Although a second extraction at 60°C. removed an additional 8% of the total nitrogen, this fraction was not included in the present studies. The zein was isolated by dialysis and lyophilization as described below: about 90% of the nitrogen extracted was precipitated as zein. Aqueous ethanol having a concentration of approximately 70% by volume was prepared conveniently by diluting 70 ml. of 95% ethanol to 95 ml. with water.

[3]Mention of firm names or trade products is for identification only and does not imply endorsement by the U.S. Department of Agriculture.

The Amberlite IRC-50, a weak cation exchange resin, was cracked to smaller particle size in a laboratory Burr mill and then ground in a laboratory hammer mill so that about 95% passed through a 35-mesh screen. The screened resin was cycled twice through the sodium and hydrogen form and finally left in the hydrogen form after washing to remove excess acid. The resin was covered with water and stored at 5°C.

The amount of zein in the effluents from the resin columns was estimated by a turbidimetric method (3) which gives a comparative but not absolute value. Nitrogen was determined by a conventional micro-Kjeldahl procedure using mecuric oxide as a catalyst. Absorbance in the ultraviolet was determined in a 1-cm. cell of a Cary recording spectrophotometer, Model 11. Electrophoresis experiments were performed with an Aminco portable electrophoresis unit in a closed 7-ml. analytical cell at 1.5°C. using 15 ma. current.

Adsorption-Desorption Procedures. The adsorption columns were packed with IRC-50 resin (hydrogen form) which had been equilibrated with 70% ethanol by mixing with four changes of 70% ethanol and allowing 10 to 20 minutes' contact each time. The equilibrated resin was slurried with 70% ethanol into the column and allowed to pack by settling. A volume of 70% ethanol equal to two or three times the holdup volume of the column was allowed to percolate through the resin to ensure equilibration. The period of contact of resin with ethanol was kept to a minimum because long periods, such as 24 hours, caused nearly complete loss of ability for the resin to adsorb zein from solution.

The zein was applied to the column as a 1 to 3% solution in 70% ethanol. For preparative runs as much as 1.4 g. of zein were used for a column 18 × 600 mm.; smaller proportions were used for chromatographic fractionation. After the column was washed with 70% ethanol to remove unadsorbed material, the adsorbed zein was removed, either in one step by elution with a 0.5*M* solution of sodium chloride in 70% ethanol, or progressively by increasing concentrations of sodium chloride up to that level.

Recovery of Dry Zein. Zein was precipitated from solution in 70% ethanol by dialysis against water at about 2°C. Solutions containing salt were first dialyzed against 70% ethanol to remove salt and thereby avoid retention of salt in the precipitated zein (levels as high as 5% salt in the zein were encountered otherwise). The dialysis against ethanol and against water was conducted as a batch operation with a sufficient number of changes of ethanol or water to ensure less than 0.2% residual salt in the first case and less than 0.1% residual ethanol in the second case.

The flocculant precipitate of zein was separated by centrifuging. The precipitate was resuspended in about ten times its volume of water and the suspension was lyophilized. In some cases the entire nondialyzable contents of the dialysis tubing were mixed and lyophilized. In either case an easily powdered product was obtained which did not require grinding and was not hygroscopic. Some mechanical loss usually occurred during lyophilization because of the ease with which the light fluffy product was carried over into the receiver by the rush of water vapors, particularly when there was any vibration of the apparatus.

Results and Discussion

Purification of Commercial Zein by Adsorption-Desorption. Commercial zein dissolved in 70% ethanol was separated into two fractions: (a) material not retained on the ion-exchange resin and (b) material eluted by 0.5*M* sodium chloride in 70% ethanol. In a typical preparation the recovery of total nitrogen was 101%; the unadsorbed material, which included the yellow pigment, accounted for 11% and the eluted fraction for 90%. The purified product obtained by dialysis of the eluted fraction against ethanol and then against water, followed by lyophilization, had a nitrogen content of 16.0% (dry basis). A 1% solution of the purified zein in ethanol was clear and very nearly colorless.

The nitrogenous material in the unadsorbed fraction was not characterized. The relatively large proportion may reflect interference of salts with adsorption of protein by the IRC-50 resin. Studies of gradient elution of zein showed that 0.003*M* sodium chloride in 70% ethanol could displace over 50% of the zein from the resin.

Purification of Unmodified Zein. The same adsorption-desorption procedure was applied to a preparation of unmodified zein having a nitrogen content of 13.5%. The unadsorbed fraction contained only 1% of the total nitrogen, whereas 86% of the nitrogen was in the fraction eluted with 0.5M sodium chloride in 70% ethanol. The nitrogen content of the resulting white powder was 16.4% and remained unchanged by a repetition of the adsorption-desorption procedure. The amount of unadsorbed nitrogenous material is very small in this case probably because of rather complete removal of salts by dialysis during zein preparation. The 13% nitrogen unaccounted for probably is material irreversibly adsorbed under the conditions used. No study was made of the nature of this portion of the nitrogenous components of the crude zein preparation, although it may correspond to beta-zein fraction, which is eliminated in the preparation of commercial zein (7).

Properties of Purified Zein. The ultraviolet absorption spectrum of a typical purified commercial zein is shown in Fig. 1, together with the

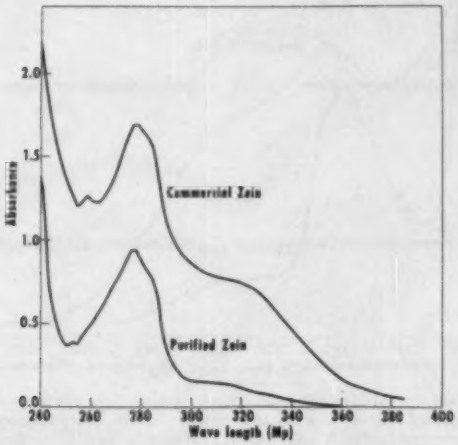

Fig. 1. Ultraviolet absorption spectra of commercial zein before (upper curve) and after (lower curve) purification by adsorption-desorption. Concentration 2 mg. zein per ml. and 1.75 mg. zein per ml. (determined turbidimetrically), respectively, in 70 volume-percent ethanol.

absorption curve of the original sample. The purified unmodified zein showed a similar ultraviolet absorption spectrum, whereas the crude unmodified zein contained larger amounts of contaminants absorbing in the ultraviolet (Fig. 2) than did the commercial zein.

The effectiveness of pigment removal is shown by the low absorbance in the region of 3,200 A for purified zein. In the region of the protein absorption peak (around 2,770 A) a strong background absorbance from pigments and other impurities in the crude zein shifts the peak upward to a considerable degree.

A quantitative comparison of the ultraviolet absorption characteristics of the crude and purified zeins is provided by the ratio of absorbance at 2,770 A (the position of the absorption maximum for purified zein) to the absorbance at 3,200 A (near the position of maximum absorption for samples of separated impurities high in the yellow pigment) and to the absorbance at 2,550 A (the position of a minimum in the absorption curve for the purified zein and the approximate position of the minimum in typical protein absorbance curves). Absorbance ratios for representative preparations before and after purification are as follows:

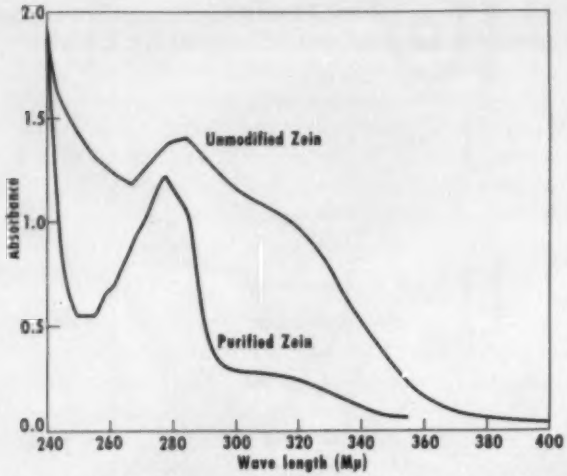

Fig. 2. Ultraviolet absorption spectra of unmodified zein extracted from corn meal, before (upper curve) and after (lower curve) purification by adsorption-desorption. Concentration 0.08 and 0.28 mg. nitrogen per ml. (the latter equivalent to about 2 mg. zein per ml.), respectively, in 70% ethanol.

	2,770 A/3,200 A		*2,770 A/2,550 A*	
	Crude	*Purified*	*Crude*	*Purified*
Commercial zein	2.3	6.8	1.4	2.5
Unmodified zein from meal extract	1.5	5.2	1.0	2.3

The marked change in the 2,770/3,200 A absorbance ratio reflects the considerable removal of pigments, although further purification appears possible for one or both samples since a difference exists between the two after purification.

Moving-boundary electrophoresis patterns for crude and purified zein are shown in Fig. 3. All zein samples were dissolved in sodium glycinate-sodium chloride buffers at pH 11.6 (5), since these conditions always gave complete solution. Zein from another corn sample was soluble in buffers at a pH as low as 10.5, possibly as a result of differences in the number of base-binding groups, such as free carboxyl groups. The use of the higher pH did not alter the electrophoretic properties of this zein. While purification had relatively little effect on the electrophoresis pattern of commercial zein, it changed that of unmodified zein considerably. The disappearance of the faster-moving component which had a mobility of about 5.5×10^{-5} cm^2/v/sec. and comprised around 50% of the total pattern area, hardly can be explained entirely by the loss of 13% nitrogen during purification.

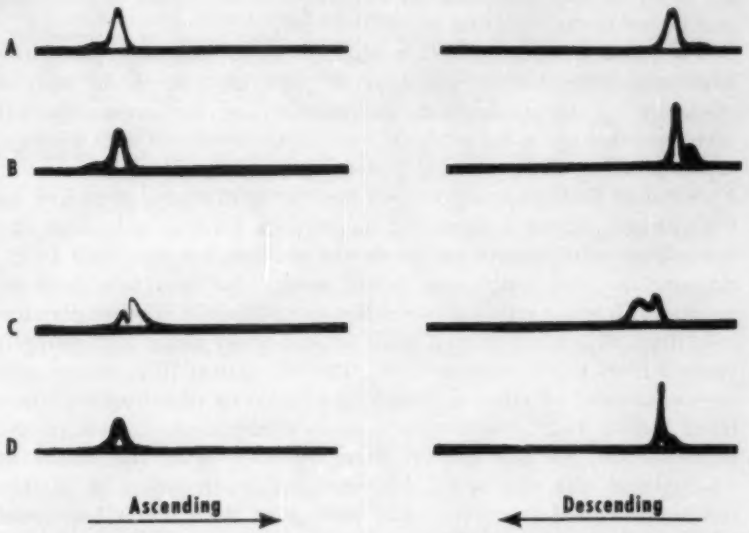

Fig. 3. Moving-boundary electrophoresis patterns of commercial zein, before (A) and after (B) purification, and of unmodified zein, before (C) and after (D) purification. Concentration of protein 0.5% in sodium glycinate-sodium chloride buffer, pH 11.6, ionic strength 0.1, 120 minutes; the delta and epsilon boundaries approximately coincide with the beginning of the patterns.

Possibly purification also removed protein-complexing impurities which altered the electrophoretic mobility of part of the zein protein. The mobilities of the main component in the samples of purified zein were in the range of 4.3 to 4.7 for both ascending and descending patterns and were in good agreement with the range of 4.0 to 4.3 reported by Mertz *et al.* (8) for the electrophoretic component which was predominantly in their zein fraction.

Solubility of the purified zein in 70% ethanol generally was complete at levels of 2 g. per 100 ml. Crude zein preparations generally were soluble to the extent of at least 85% after precipitation by dialysis and lyophilization. Occasional difficulties in redissolving the crude zein were attributed to leaving too high a level of ethanol (1 to 2%) in the solution when the precipitated zein was lyophilized with the entire contents of the dialysis bag.

Purification of zein for animal feeding studies might be done advantageously by the procedure described here. Not only the purity but also the physical properties of the product should be beneficial. Maximum digestibility of the protein would be expected as a result of

the finely divided state and the absence of denaturation or horniness as reflected in the solubility in 70% ethanol.

Purification Based on Solubility. Prior to the development of the adsorption procedure the possibility of purifying zein on the basis of solubility differences between the protein and the impurities was investigated. Among the methods tried were: extraction with solvents, reprecipitation, and prolonged dialysis against 70% ethanol.

None of these procedures were entirely satisfactory, as judged by the nitrogen content and color of the product. Ether extraction of one unmodified zein preparation raised the nitrogen content from 13.5% nitrogen to 14.6% nitrogen; while some color was removed, the product still was a yellow powder. Reprecipitation of another preparation from 70% ethanol by dialysis against water raised the nitrogen content from 13.5% only to 13.7%. Dialysis against 70% ethanol was somewhat more effective, as shown by an increase of nitrogen content from 13.5 to 15.2%, with simultaneous elimination of most of the pigmentation. Variable results were encountered in the extent to which color was eliminated during dialysis; some sizes of dialysis tubing permitted relatively rapid passage of the colored impurities while others permitted only slow removal or none at all. No attempt was made to determine whether this result was caused by differences inherent in tubing size or its age.

Gradient Elution of Zein. Since column chromatography has been used for fractionation of proteins, some trials were made of fractional elution of zein from IRC-50 resin columns. Sodium chloride gradients from 0 to 0.005*M* in 70% ethanol gave elution patterns that varied from a broad peak to partial resolution into two or more peaks and accounted for 50% or more of the adsorbed protein. A further quantity of protein was desorbed by raising the sodium chloride concentration in one step to 0.2*M* or 0.5*M*. Neither this final fraction nor the one from gradient elution appeared to be homogeneous, since rechromatography gave elution patterns similar to those for the original zein preparation. The results are suggestive of association-dissociation behavior comparable to that attributed to zein by Scallet (9) on the basis of fractional precipitation and electrophoresis studies.

Acknowledgment

The authors wish to thank Eugene H. Melvin and Curtis A. Glass for their advice and assistance with the spectrophotometric analyses.

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ORGANIC ACIDS OF BARLEY GRAIN[1]

J. S. WALL, L. C. SWANGO, D. TESSARI, AND R. J. DIMLER

ABSTRACT

The identity and amounts of organic acids contained in whole barley grain and in barley grain fractions were investigated, since they may play significant roles in germination and seed metabolism. The relative efficiency of ion-exchange adsorption compared to ether extraction as a means of isolating the acids from alcoholic extracts of the grain was determined. An improved procedure of partition chromatography on silica gel was developed to separate and determine the acids. Paper chromatography was employed to identify individual acids and to make preliminary qualitative studies of the acids in the extract. Most of the organic acids in barley extracts were accounted for as malic, acetic, succinic, fumaric, malonic, alpha-ketoglutaric, lactic, citric, aconitic, and pyrrolidone carboxylic. The reported presence was confirmed of a number of phenolic acids contained in barley, including ferulic, vanillic, *p*-hydroxybenzoic, and *p*-coumaric.

There is evidence that certain organic acids in cereal grains play an important role in determining ease of seed germination. Täufel and Pohloudek-Fabini (21) report that a positive correlation exists between viability and citric acid content of stored seeds of several species, including barley. They attribute this correlation to the stimulation Krebs cycle acids give aerobic respiration. In contrast, an inhibitory action upon barley germination was demonstrated by Cook and Pollach (6) to be due to some aromatic acids in the grain. Only vanillic acid was characterized among these substances.

[1] Manuscript received November 7, 1960. Contribution from the Northern Regional Research Laboratory, Peoria, Illinois. This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

There has been little information available pertaining to the nature and amounts of the organic acids in barley grains, including those of metabolic importance. While the present work was in progress, Houston and Kester (11) presented a preliminary report of analyses for various organic acids in barley and in a number of other cereal grains. Van Sumere and his associates (22) recently identified several phenolic acids contained in barley husks.

A study was undertaken to develop improved methods for the isolation, identification, and determination of the various organic acids of barley grain. Pearling was employed to separate the husk fraction of barley to obtain greater concentration of the acids characteristic of this portion of the grain. Distribution of all the barley acids was studied in the fractions obtained by pearling because of the importance of pearled barley as a food.

Materials and Methods

Samples for Analysis. The samples of Tregal variety barley employed in these studies were obtained from the 1957 crop grown at the North Dakota Agricultural Experiment Station. The grain was received shortly after harvesting and stored at 4°C. Some older grain of the same variety and source which had been stored under continuous refrigeration was also submitted to a cursory study to note any effects of this type of storage. Pearling, as carried out on a Strong-Scott laboratory mill [2] for 2 minutes, removed most of the husk and much of the embryo. The pearlings represented 14.5% of the weight of the initial grain.

Ethanol Extraction of Barley. By means of a large Waring Blendor (Model CB3), 300 g. of barley, pearled barley, or pearlings were macerated together with 900 ml. of 80% ethanol for a period of 15 minutes. During this time the blender speed was increased, at intervals, to the maximum; the temperature of the extracting solution rose almost to boiling, thereby favoring extraction and enzyme inactivation. The suspension was then cooled and centrifuged. The insoluble residue was re-extracted twice with 800-ml. volumes of 80% ethanol in the Waring Blendor. The extracts were combined. The pH of the extracts was 5.5; for extracts to be run through ion-exchange columns, sufficient alkali was added to bring the pH to 7.2. The solutions were then concentrated under vacuum to a volume of about 30 ml. Insolubles were removed by centrifugation.

Ion-Exchange Treatment. One procedure investigated for separat-

[2] Mention of firm names or trade products is for identification only and does not imply endorsement by the U.S. Department of Agriculture.

ing the organic acids in the extract of the barley from the carbohydrates and other substances that might interfere with subsequent chromatography was adsorption of the acids on an ion exchanger as described by Resnik *et al.* (17). The extract was passed through a 1.2 by 25-cm. column of Dowex 1 × 8 (50–100 mesh) in the carbonate form. The column was then washed with 200 ml. water and the acids were eluted with 500 ml. of 1.5*N* ammonium carbonate. After vacuum concentration of this effluent almost to dryness to remove ammonium carbonate, the residue was made up to a volume of 1 ml. with water.

Ether Extraction of Barley Acids. Another method tested for recovering the organic acids from the aqueous concentrate of the initial alcohol extract of barley was based on ether extraction. The aqueous solution was adjusted to pH 1 by careful addition of 9*N* sulfuric acid and transferred to a liquid-liquid extractor (Kutscher-Steudel) of 50-ml. capacity. After extraction with 100 ml. of ether for 72 hours, the ether solution was twice extracted with 150 ml. of 2% sodium bicarbonate solution. The combined bicarbonate solutions were neutralized and concentrated to 30 ml., acidified to pH 1, and again extracted for 72 hours with ether. After removal of the ether, the acids were made up to 1 or 2 ml. in water or 50% acetone solution.

Paper Chromatography. Paper chromatography was used to obtain preliminary information about the number, kind, and relative quantities of organic acids in the extracts. Two-dimensional chromatography similar to that described by Cheftel *et al.* (5) separated most of the nonvolatile aliphatic acids. The initial descending irrigation on Whatman No. 1 paper, 46 by 57 cm., in the long direction was with 95% ethanol, concentrated ammonium hydroxide, and water (8:5:15) for 24 hours. The terminal edge of the paper was serrated to allow the solvent to run off uniformly. A solution of n-butanol, formic acid, and water (120:20:20) was employed as the solvent in the second direction for 18 hours, and it also was used extensively in unidirectional chromatograms for characterizing substances eluted from chromatographic columns. After drying, the papers were examined under ultraviolet light with a lamp having emission maximum at 260 mμ, to detect aromatic acids and such unsaturated acids as fumaric and aconitic. After the papers were sprayed with semicarbazide reagent (14), keto acids, such as pyruvic and alpha-ketoglutaric, were detected under the ultraviolet light. The papers were next sprayed with bromcresol green, 40 mg. in 100 ml. 95% ethanol adjusted with alkali to a green color, to develop spots for acids.

The aromatic acids were separated by descending chromatography in a two-dimensional system consisting of 95% ethanol and 2*N*

ammonium hydroxide (9:1) in the initial direction for 18 hours and tertiary amyl alcohol, water, and glacial acetic acid (6:94:0.5) in the second direction for 6 hours (6). Spots were detected by ultraviolet light and characteristic fluorescence or absorbance noted. The papers were also sprayed with diazotized *p*-nitroaniline and colors were noted before and after an additional alkaline spray (20). Volatile organic acids were separated by the paper-chromatographic procedure of Kennedy and Barker (13).

Column Chromatography. Separation of organic acids was based on a combination of many reported modifications of the original Isherwood (12) technique of partition chromatography on silica gel columns. The silica gel was prepared from Mallinckrodt analytical grade 100-mesh silicic acid as described by Bulen, Varner, and Burrell (2). Ten grams of the dried silica gel were ground together with 6 ml. of 0.5*N* sulfuric acid. Chloroform saturated with 0.5*N* sulfuric acid, 30 ml., was added to form a thin slurry after thorough stirring. The slurry was poured into a glass tube, 1.0 by 50.0 cm., fitted near the end with a porous glass disk and terminated with rubber tubing and screw clamp to regulate solvent flow. The silica gel was packed in the tube under 2 lb. pressure of nitrogen. The samples were introduced to the column in aqueous solution by the method of Zbinovsky and Burris (24). A circular pad of filter paper cut from sheets 2-mm. thick was applied to the silica gel surface under a layer of chloroform saturated with 0.5*N* sulfuric acid. The chloroform was then run through the column to the disk surface and the glass tube's inner surface wiped with a cotton swab. Then 0.2 ml. of 9*N* sulfuric acid followed by 0.3 ml. of extract and 0.1 ml. of 9*N* sulfuric acid were added in that order to the surface of the disk. This aqueous solution was barely allowed to enter the column and 3 ml. of chloroform saturated with 0.5*N* sulfuric acid were placed above the disk. The rubber tubing was removed, and the column was transferred to the fraction collector and connected to the solvent reservoir as illustrated in Fig. 1. The solvent reservoir had previously been placed in position and filled as described below.

Elution of the acids with good resolution and recovery was achieved by a modification of the continuous gradient elution procedure of Donaldson *et al.* (8), employing an all-glass apparatus (Fig. 1). The arm of the lower vessel was just below the 350-ml. capacity level. In the lower container were placed 390 ml. of chloroform saturated with 0.5*N* sulfuric acid; in the upper chamber, 350 ml. of 20% tertiary amyl alcohol-80% chloroform saturated with 0.5*N* sulfuric acid. With stopcock *A* closed and clamp *C* and stopcock *B* open, elution with only

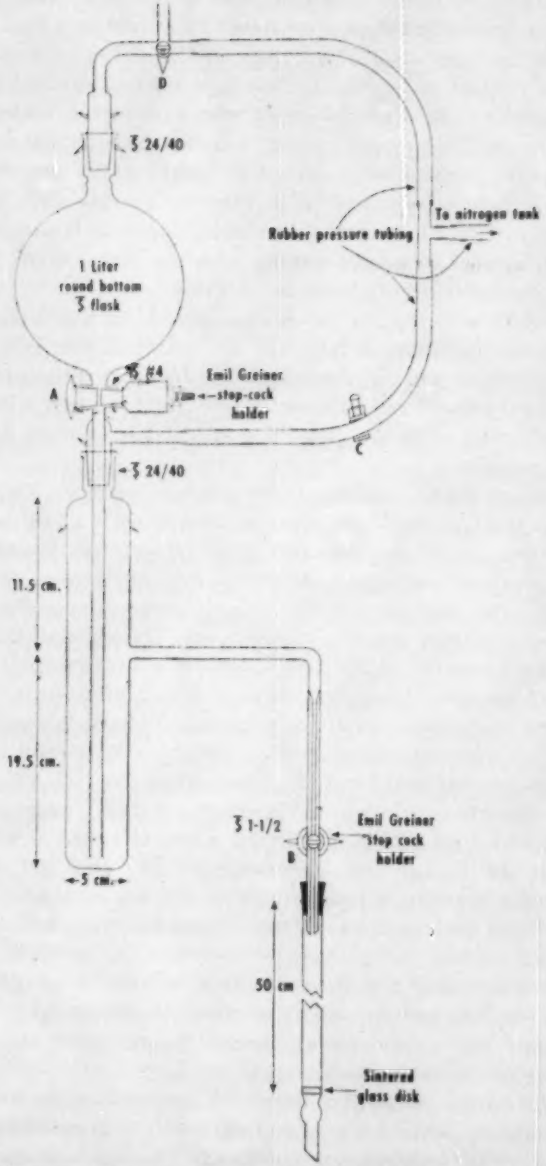

Fig. 1. Apparatus for gradient elution of organic acids during partition chromatography on silica gel.

the chloroform was begun with collection of fractions. The nitrogen pressure was gradually elevated to about 2 lb. so that a flow rate of 0.75 ml. per minute was attained. After 40 ml. were collected, stopcock *A* was opened and clamp *C* closed, permitting gradual mixture of the contents of the upper chamber with that in the lower. When the contents of the upper chamber were exhausted, 350 ml. of a solution of 60% tertiary amyl alcohol-40% chloroform saturated with 0.5*N* sulfuric acid were placed in it. Pressure was maintained in the lower vessel during this operation by closing stopcocks *D* and *A*, which were again opened to permit mixing with the new solvent. Elution was then continued overnight at the previous rate.

Determination of Eluted Acids. To determine the acids of the column effluent by titration, fractions of 2 ml. each were collected by means of a drop count automatic collector in tubes containing 4 ml. of an aqueous phenol red solution. These were titrated with 0.01*N* sodium hydroxide while the two-phase system was agitated by means of a jet of nitrogen.

An accurate determination of the aromatic acids in the column effluent was not possible by titration because of their small concentration. However, more sensitive ultraviolet absorption measurements could be employed for their detection and analysis as shown by Sondheimer (19). For this purpose 4-ml. fractions were collected and the aqueous indicator solution was omitted. The absorption of these fractions was measured at 260 and 320 $m\mu$ in a Beckman DU spectrophotometer against chloroform saturated with 0.5*N* sulfuric acid as a blank. Even after redistillation, the reagent-grade tertiary amyl alcohol exhibited considerable absorbance at 260 $m\mu$ which resulted in an increasing background absorbance of the effluent at that wave length. Therefore the absorbance values of effluent fractions from a control column to which no extract sample was added was used as a blank to correct for the background absorbance at 260 and 320 $m\mu$. The absorbance of standard acid solutions at 260 $m\mu$ in similar solvents (Table I) was used to calculate the amounts of phenolic acids in the effluent.

Characterization of Eluted Compounds. In addition to the position of elution from the column and paper chromatography, specific chemical reactions and ultraviolet absorption spectra were employed to characterize the separated barley acids.

The ultraviolet absorption spectra of aromatic acids were determined on fractions containing peaks from columns upon which the UV absorption of the effluent was measured. The spectral curves were obtained with a Cary automatic recording spectrophotometer employ-

TABLE I

ULTRAVIOLET ABSORPTION CHARACTERISTICS OF AROMATIC ACIDS IN BARLEY

ACID	$\lambda_{max.1}$[a]	$\lambda_{max.2}$[a]	$\lambda_{min.}$[a]	E $_{max.1}$[b] $\times 10^{-4}$	E 260[b] $\times 10^{-4}$	E 320[b] $\times 10^{-4}$
	mμ	mμ	mμ			
Ferulic	320	295	262	1.92	0.25	1.92
Vanillic	260	290	..	1.18	1.18	0.00
p-Coumaric	310	...	257	2.06	0.18	1.65
p-Hydroxybenzoic	255	.	240	1.48	1.40	0.00
Unknown A	280	310	260	....	....	....
Unknown B	260	310	275	....	....	....
Unknown F	272	...	...	....	....	....
Unknown G	260	...	245	....	....	....

[a] Determined upon effluent fractions from column.
[b] Determined upon knowns in chloroform saturated with 0.5*N* H_2SO_4.

ing as blanks analogous fractions from the control column used for UV background correction.

For paper chromatography the fractions from a column containing a peak were combined and dried under vacuum. Nonvolatile acids present as sodium salts after titration were taken up in small volume (0.2–0.5 ml.) of 0.1*N* sulfuric acid before application to the paper. Certain of the free aromatic acids were dissolved in acetone.

Standard Acids. The standard acids employed for both column and paper chromatographic studies were from commercial sources and were recrystallized when necessary. Some of the phenolic acids tested were obtained from A. N. Booth of the Western Utilization Research and Development Division, U.S. Department of Agriculture. Compounds synthesized in our laboratory by established procedures, as indicated, are pyrrolidone carboxylic acid (9) and *p*-coumaric acid (16).

Phosphorus Determination. Phosphorus was determined by a modification of the method of Allen (1).

Results

Ion-Exchange Adsorption. The passage of the alcoholic extract of barley or a known mixture through the column of Dowex 1 resin resulted in almost quantitative removal of acidic substances (Table II). Only 3.7% additional acids were recovered by a passage of the washings from the column through a second anion exchanger of similar dimensions. The passage of additional ammonium carbonate solution through the column after elution resulted in only 2.6% additional recovery of acids. However, the rapidity of elution of individual acids with ammonium carbonate varies, depending upon their structure, so that certain ones were preferentially retained. As evidenced from the paper-chromatographic studies, phenolic acids were absent from the

TABLE II

ADSORPTION, RECOVERY, AND COMPOSITION OF BARLEY ACIDS PASSED THROUGH ANION EXCHANGER (DOWEX 1 × 8, 50–100 MESH CARBONATE FORM) (1.2 BY 2.5 CM.)

COMPONENT	ACID FROM 300 g. BARLEY	
	meq.	%
Acid not adsorbed on anion exchanger (determined by adsorption on second column)	0.28	3.7
Acid eluted by 500 ml. 1.5*N* $(NH_4)_2CO_3$	7.10	94.0
Acid eluted by additional 500 ml. 1.5*N* $(NH_4)_2CO_3$	0.20	2.6
Total acid substances in extract	7.58	100.0
Total organic acids determined by column chromatography	3.32	43.6
Inorganic phosphorus	1.66	22.2
Organic phosphorus	0.33	4.4

ammonium carbonate eluate. They could be recovered from the column by elution with a solution of 80% ethanol, 3.0*N* in acetic acid, although this disrupted the basic column. The use of ammonium carbonate as eluent resulted in greater recovery of volatile acids during the final extract concentration than could be obtained using organic acids as eluents.

The titratable acidity of the ion-exchange extract gave a measure of other anions present in the extract in addition to organic acids. Thus only 44% of the total acid of the extract was determined as organic acids upon chromatography, as shown in Table II. Of the remaining acidic material about half was determined to be inorganic phosphorus. Other inorganic anions were evidenced in two-dimensional paper chromatograms.

Ether Extraction. The ether extract contained less inorganic ions and other hydrophilic materials that interfered with the chromatography than did the extract purified by ion exchange. The two-dimensional paper chromatograms indicated many inorganic ions were absent and allowed acid spots to be more sharply defined (Fig. 2). However, losses of the more polar organic acids are also possible during ether extraction. Extraction of known quantities of citric acid from an acid aqueous medium revealed that the 72-hour extraction recovered only 91% of the citric acid under the conditions employed. Much poorer citrate recoveries were obtained during shorter extraction times.

The bicarbonate extraction eliminated many nonacid, ultraviolet-absorbing, ether-soluble substances, such as other phenols, which would also give positive reactions on papers with the diazotized *p*-nitroaniline reagent. The extract contained several phenolic acids (Fig. 3). The four major phenolic acids are probably identical with

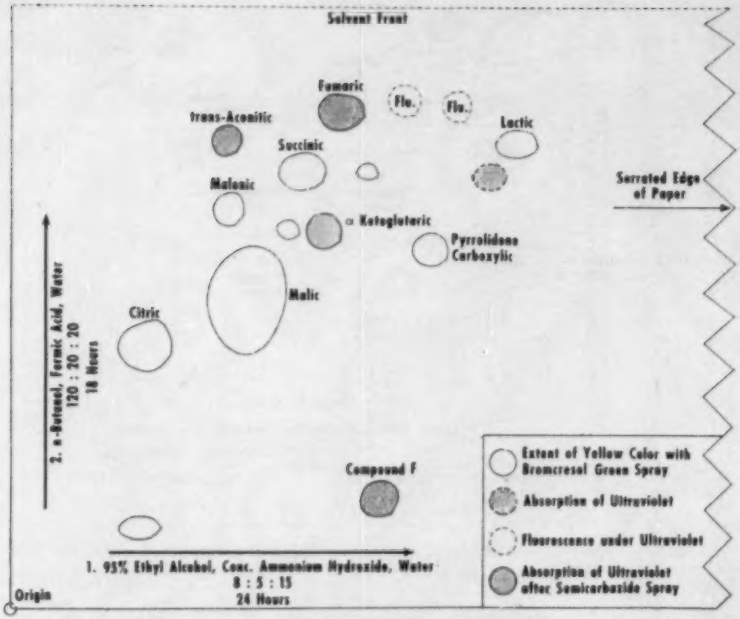

Fig. 2. Two-dimensional paper-chromatographic separation of nonvolatile acids of ether extract of barley. Approximately 60 μl. of concentrate applied.

those found by Cook and Pollach (6). In addition to vanillic acid identified by those workers, *p*-coumaric, ferulic, and *p*-hydroxybenzoic acids were characterized on the papers by R_f's, UV absorption, and reaction colors with diazotized *p*-nitroaniline, as indicated in Fig. 3.

Column Chromatography. Figure 4 shows a typical separation of the organic acids in an ether extract of whole-grain barley as determined by titration of the effluent fractions with standard alkali. The results of measuring the UV absorption of the effluent fractions to detect and determine the aromatic acids are shown in Fig. 5. Qualitatively similar resolutions were obtained with the other extracts. The positions of elution of the individual acids were closely duplicated in different runs with each of the extracts and with known mixtures.

The identity of the peak due to malonic acid in Fig. 4 was further verified by a unique green color that it yielded with diazotized *p*-nitroaniline reagent on paper chromatograms. The pyrrolidone carboxylic acid from the column effluent was converted by hydrolysis with 6*N* hydrochloric acid for 24 hours to glutamic acid, which was identified also by paper chromatography.

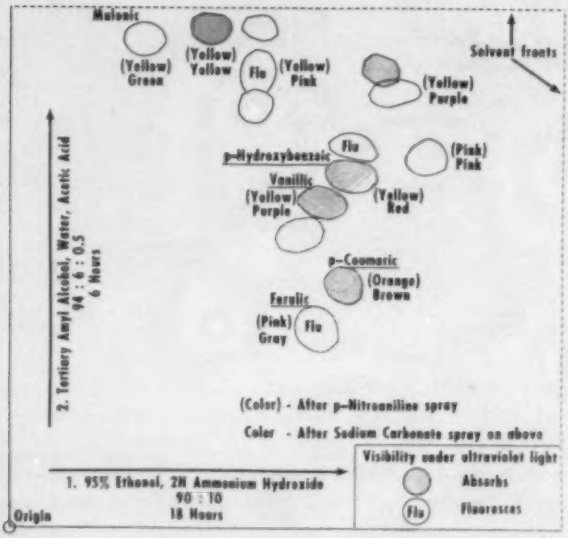

Fig. 3. Two-dimensional paper-chromatographic separation of phenolic acids of barley. Approximately 60 μl. of concentrate from ether extract of whole barley applied.

Some of the acid peaks found in ether extracts have not been positively characterized and are designated by letter. The UV absorption characteristics of certain of these substances are tabulated in Table I.

Aconitic, malonic, and pyrrolidone carboxylic acids were eluted in close proximity (Fig. 4). Vanillic and ferulic acids were not well resolved, as is also the case for *p*-hydroxybenzoic and acetic (Figs. 4 and 5). However, each of the phenolic compounds can be detected and analyzed independently by utilizing their characteristic UV absorptions as listed in Table I. The cinnamic acid derivatives, ferulic and *p*-coumaric acids, were determined by absorption at 320 mμ; vanillic and *p*-hydroxybenzoic acids were determined at 260 mμ.

Recoveries of individual acids applied in known quantities to the columns were quantitative (greater than 95%). This recovery indicated that little esterification of the acids occurred during their exposure to the eluting solvent.

Amounts of Acids in Extracts. The data in Table III, which summarizes the amounts of each of the identified acids contained in the various extracts, represent the averages of duplicate analyses of at least two extractions.

Malic acid is the preponderant organic acid present in barley. It

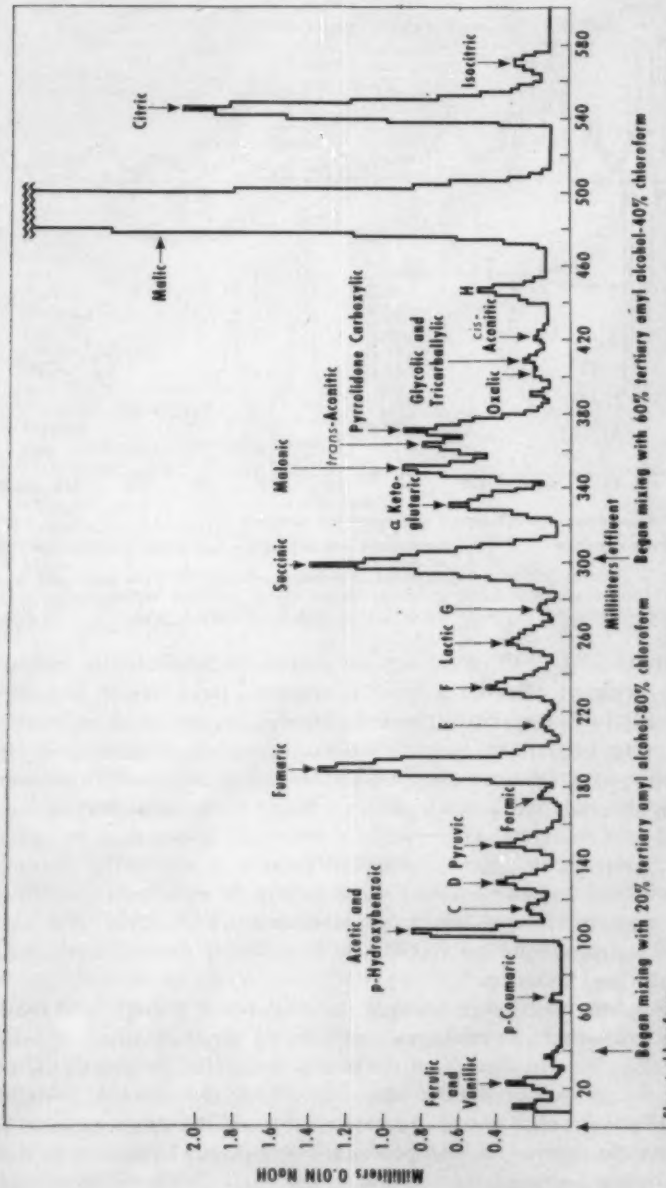

Fig. 4. Determination by titration of organic acids separated from an ether extract equivalent to 90 g. of whole barley upon partition chromatography on a silica gel column. All eluents were saturated with 0.5*N* sulfuric acid.

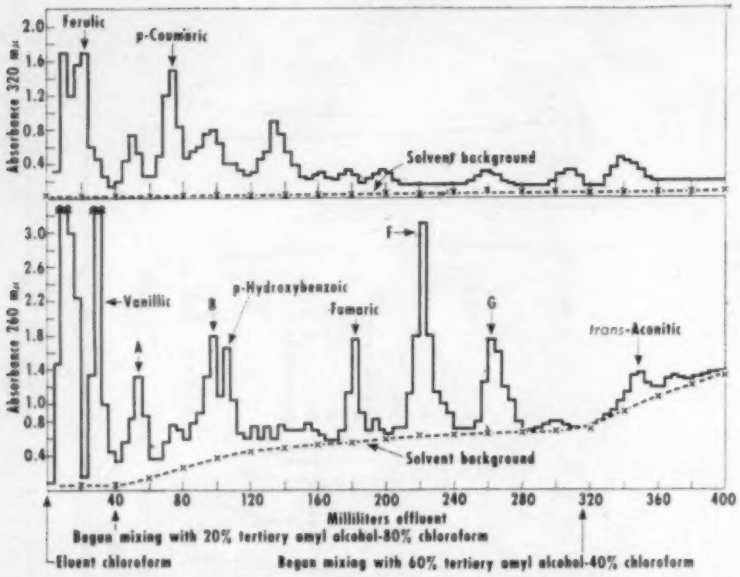

Fig. 5. Determination by ultraviolet absorbance of organic acids separated from ether extract equivalent to 90 g. whole barley upon partition chromatography on silica gel column. All eluents were saturated with 0.5*N* sulfuric acid.

constitutes 60% of the total organic acid of the whole-barley extracts. Other acids of the Krebs or "tricarboxylic acid" cycle including citric, succinic, fumaric, alpha-ketoglutaric, isocitric, and *cis*-aconitic acids were present in lesser amounts. The *trans*-aconitic acid was probably produced from the less stable biologically active *cis*-isomer during the isolation procedure. Only small amounts of the *cis* compound were recovered. Other acids of metabolic importance, including acetic, pyruvic, and lactic, were also present in the barley extracts. Malonic acid and compound F were present in significant quantities. Only traces of formic, oxalic, glycolic, and tricarballylic acid were detected. Pyrrolidone carboxylic acid is probably derived from glutamine during extraction.

Some differences occur between the amounts of a single acid found in the ion-exchange extract, as compared to those obtained by ether extraction. The quantities of citric acid were slightly greater in the ion-exchange extract than in the ether. This difference is probably due to poorer recovery of the polar acids in the ether process. In contrast, the ether-extraction procedure yields more fumaric acid than does the ion exchange.

TABLE III

ORGANIC ACIDS OF BARLEY AND BARLEY PEARLING FRACTIONS

METHOD USED TO DETERMINE ACID	WHOLE BARLEY		PEARLED BARLEY ETHER EXTRACT	BARLEY PEARLINGS ETHER EXTRACT
	Ion-Exchange Extract	Ether Extract		
	μeq/100 g	μeq/100 g	μeq/100 g	μeq/100 g
UV Absorption				
Vanillic	....	2.4	0.6	16.3
Ferulic	....	0.9	0.4	5.4
p-Coumaric	....	1.0	0.1	6.8
p-Hydroxybenzoic	....	0.9	0.2	5.1
Titration				
Acetic	62.5	29.2	21.2	35.2
Pyruvic	7.1	8.1	....	....
Formic	4.0	3.6	....	....
Fumaric	40.5	58.3	49.3	115.0
Compound F	....	19.2	....	....
Lactic	14.9	16.7	12.0	42.7
Succinic	78.0	82.2	63.7	181.5
Alpha-ketoglutaric	30.8	40.0	32.5	75.0
Malonic	22.0	25.5	18.6	48.3
trans-Aconitic	16.0	17.9	14.2	20.1
Pyrrolidone carboxylic	25.4	30.5	20.3	40.4
Oxalic	2.1	2.0	....	....
Glycolic	3.5	2.5	....	....
Tricarballylic	3.5	2.5	....	....
cis-Aconitic	4.0	3.5	....	....
Malic	875.3	822.0	533.0	1400.0
Citric	100.5	80.7	30.4	170.0
Isocitric	10.3	8.7	....	30.8

Data of Table III establish that the phenolic acids of barley are primarily concentrated in pearlings. Studies by Van Sumere *et al.* (22) indicate that they are associated with the husk. Barley pearlings contained appreciably greater amounts of all the organic acids on a weight basis than did the whole grain. However, the pearled barley, which is primarily endosperm, contained a greater total amount of organic acid.

Discussion

Silica Gel Chromatography. The column-chromatography procedure described affords numerous advantages over earlier methods in simplicity, resolving power, and rapidity. A system for the separation of organic acids on partition columns employing gradient elution with increased resolving power also has been described recently by Dajani and Orten (7). With an initial 350-ml. volume of chloroform in the mixing flask, satisfactory resolution of lactic and succinic acids or of certain aromatic acids was not achieved with an initial tertiary amyl alcohol concentration of greater than 20% in the upper flask. The use of a second gradient approaching 60% tertiary amyl alcohol was introduced to obtain more rapid and compact elution of the more

polar acids. The volume of 20% tertiary amyl alcohol-80% chloroform employed prior to changing to the 60% tertiary amyl alcohol-40% chloroform is critical with respect to the elution sequence and resolution of alpha-ketoglutaric, aconitic, malonic, and pyrrolidone carboxylic acids. Variations in position of elution of these acids resulting from changes in solvent schedule may be used to advantage to obtain better resolution of certain of the acids.

Extraction. In some experiments 80% acetone was used instead of 80% ethanol to extract barley. The greater volatility of the acetone proved undesirable. The data obtained with the acetone extracts were similar to those with the alcohol extracts, indicating that little esterification of organic acids occurred during extraction with the ethyl alcohol solution. Oxalic acid which might be present as the insoluble calcium salt at the pH of the barley macerate may not be completely extracted by the 80% ethanol.

The two isolation procedures, ion-exchange adsorption and ether extraction, served as a check on the efficiency of each. Ease of recovery of individual acids by either procedure depends upon their relative polarity. Ion-exchange chromatographic separation of organic acids developed by Busch *et al.* (3) gives evidence that malic acid is more readily eluted from the anion exchanger Dowex 1 than fumaric acid is with polar solvents, and that polarity of the acid is important in determining the elution rate. Data in Table II indicate that little additional acids are recovered by doubling the elution volume of ammonium carbonate. However, the retained acids are predominantly the less polar acids which are more highly bound by adsorption to the aromatic resin. Carrol (4) has shown that elution with organic solvents speeds the recovery of fumaric and aromatic acids from ion-exchange resins relative to more polar acids.

The time of liquid-liquid extraction of the aqueous extract with ether should be governed by the time required for removal of the most polar acid component, such as citric. The efficiency of removal of citric acid or similar polar acids should be determined for each Kutscher-Steudel apparatus and the time of extraction of organic acids established accordingly.

Significance of Acid Concentrations. The large quantity of malic acid suggests that the further metabolism of this acid to oxalacetate is rate-limiting during the development of the seed. The accumulation of malic acid may serve as a reservoir for four-carbon units essential to the oxidation of carbohydrate and lipid via the Krebs cycle during seed germination. The other Krebs-cycle acids in barley may serve similarly. The positive correlation observed by Täufel and Pohloudek-Fabini

(21) between citric acid concentration and seed viability may not be specific, because they give no indication as to the existence or absence of any relationship between viability and the presence of other organic acids in the seed.

Elliot (10) also found significant quantities of malonic acid in germinating barley. Only recently, the widespread occurrence of malonate in plant tissues has been demonstrated, and the pathways for its enzymatic utilization and synthesis have been described by Shannon *et al.* (18). Possibly this organic acid plays an important role in plant tissue metabolism.

The phenolic acids were found primarily in the husk-containing fraction. Some have been related to lignin formation (15). Vanillic acid has been shown to delay barley germination, but the other phenolic acids in barley have not been studied relative to their role in this phenomenon. The aromatic acids appear to have wide influences on metabolism and have been shown to be active in combating the development of fungi involved in certain diseases in grain (23).

Fractionation of barley by pearling the grain proved advantageous for attaining the greatest concentration of phenolic acids. The higher concentration of other organic acids in the pearling was probably related to the fact that most of the embryo was present. The pearling process did not dissociate the barley into discrete morphological fractions, so that conclusions as to the exact anatomical distributions of organic acids cannot be deduced.

Only minor differences were found in organic acid distributions in barley of the same variety and from the same source but harvested in different years, or in the barley that had been stored in the cold for several years. No studies were made of changes that might occur in the organic acids of grain stored commercially. The techniques described in this report should facilitate the study of changes of organic acids in barley and other grains during storage and germination.

Acknowledgment

The authors wish to thank Curtis A. Glass for assistance in determining the ultraviolet absorption spectra, and Francis J. Castle for aid in the design of the glassware employed for solvent reservoirs.

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GAS PLASMA IRRADIATION OF RICE
I. Hydration Characteristics[1]

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ABSTRACT

Milled, whole-grain rice, that was subjected to a gas-plasma irradiation, established by a 60-cycle power source, at a pressure that was low enough to permit the generation of a (glow) discharge across electrodes, showed a marked increase in the amount of water that could be absorbed. A study of the effect of the major operating variables revealed: 1) with the time and pressure constant at 5 minutes and 2 mm. of mercury respectively, maximum changes in hydration characteristics occurred at about 175 ma. for Zenith variety and 150 ma. for Bluebonnet 50 rice; 2) within the pressure range of 2 mm. to 8 mm. of mercury with a current of 25 ma. to 75 ma. for 5 minutes, pressure during treatment had no great effect upon changing the water absorptive capacity; 3) at 50 ma. and 2 mm. of mercury pressure, any increase in treatment time over 45 minutes for Bluebonnet variety and 70 minutes for Zenith was inefficient in increasing the amount of water absorption.

The difference between the water-holding capacity of irradiated and nonirradiated vacuum-treated controls may possibly be explained by the wide divergence between the temperatures attained during their preparation.

There has been a very great and widespread interest in the quality of milled rice (1,2,6,8). One of the most important rice quality factors is the speed and extent of hydration that occurs upon immersion in water. To a great extent, this influences cooking time and texture of the prepared product. The effects of storing (5), variety and heating (7), freezing (9), and parboiling (3) upon hydration have been studied extensively. Stone (13) recently reported on a low-energy radiation procedure that greatly increased the water absorbancy of cotton, linters, and fibers. Similar changes were shown earlier (4) for soybeans. Brown *et al.* (4) speculated on the possibility of using irradiation to shorten the cooking time required for dehydrated seeds used as foods. The present investigation reports on the three most important operating variables of gas-plasma irradiation and their effects on the hydration characteristics of two varieties of rice. These include time and intensity of irradiation and the range of pressures used in the treatments.

Materials

Bluebonnet 50 and Zenith varieties of rice, 1958 crop of foundation

[1]Manuscript received October 13, 1960. A joint contribution from the Southern Utilization Research and Development Division and the Agricultural Engineering Research Division, Agricultural Research Service, U.S. Department of Agriculture.

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seed, were obtained near Crowley, Louisiana. Combined rice was dried immediately after harvesting, under ambient conditions, and stored in sealed cans at about 15°C. The rice was milled and whole grains were separated by standard methods (10,11,12). Glow discharge equipment was similar to that recommended by Brown *et al.* (4). The irradiation chamber consisted of a borosilicate glass tube, 51 mm. o.d. by 61 cm. long. At each end of the tube, a black iron coupling, ¾ in. nominal diameter, connected to a rubber stopper by a black iron nipple, was used for an electrode (Fig. 1). This type of metal was used to minimize sputtering of the electrodes during the treatments. High voltages were

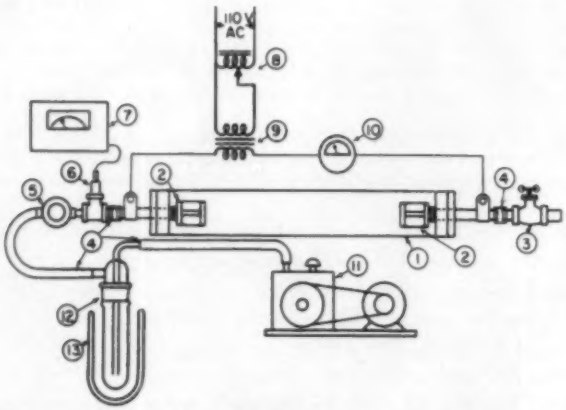

Fig. 1. Schematic drawing of gas-plasma irradiation apparatus: 1, glass tube; 2, black iron couplings (electrodes) mounted in rubber stoppers by means of short nipples; 3, needle valve; 4, rubber pressure tubing; 5, two-way solenoid valve; 6, vacuum gage tube or sensing element; 7, absolute pressure indicator; 8, variable transformer; 9, luminous-tube transformer; 10, AC milliammeter; 11, vacuum pump; 12, freeze-trap; 13, Dewar flask.

obtained from luminous-tube transformers. For currents in the range of 50 to 145 ma., a transformer rated at 6000 v. and 120 ma. (Jefferson Electric Co., Bellwood, Ill.)[4] was used. For lower intensities a 5000-v., 60-ma. source was utilized; whereas two of the larger transformers, connected in parallel or a 12,000-v., 15 kva. transformer (Westinghouse) were employed for higher intensities. The current was regulated by connecting the primary side of the transformer to a variable transformer, such as a Powerstat.

[4] Mention of trade names, equipment, or suppliers does not constitute endorsement by the Department of Agriculture over others not mentioned.

Methods

A 30.0-g. sample of rice was uniformly spread along the bottom of the treatment chamber. Rubber stoppers containing the electrodes were used to seal the ends of the treatment chamber, and other required equipment was connected as indicated in Fig. 1. Zero time was recorded and irradiation was initiated as soon as the desired pressure was established. When the vacuum controls were prepared without being irradiated, the same timing procedure was used. The treated samples, i.e., both the irradiated and the nonirradiated vacuum-treated rices, were placed in sealed containers and stored at room temperature.

There were three major sets of experiments on the effects of operating variables – current intensity, pressure in the system, and treatment time – on the hydration characteristics of whole-grain milled rice. For the study of intensity, a pressure of 2 mm. of mercury and a treatment time of 5 minutes were kept constant; for pressure, 50 ma. and 5 minutes were constant; and for time, the intensity and pressure were maintained at 50 ma. and 2 mm. of mercury.

The water-absorption values for the treated rices were determined according to a previously described procedure (7). The rice and water were first held at room temperature (about 24°C.) for 30 minutes before they were immersed in a water bath at 90°C. for 10 minutes. These values were also obtained at 70°C. Since they were generally of a lower magnitude but parallel to those for 90°C., only the latter are reported. Water absorption values are expressed on a dry-weight basis and represent the average of at least two determinations.

$$\text{Water absorption (\%)} = \frac{\text{weight of moisture in cooked rice}}{\text{weight of dry material in cooked rice}} \times 100$$

The values reported for total solids lost to the cooking water were obtained by difference from a materials balance. In conjunction with these calculations, moisture contents of the original milled rice samples were determined by the AMS method (14).

Results

Current Intensity. There was a progressive increase in amount of water absorption of Zenith variety rice, as the irradiation intensity was increased up to about 175 ma. (Fig. 2). The loss of total solids to the treating water generally paralleled the changes in water absorption up to a current of 110 ma. Thereafter the solids loss diminished as the intensity increased. This change apparently was related to the texture of the hydrated rice: thus when the solids loss decreased there was less cohesiveness between the cooked grains. Based upon hydration, the

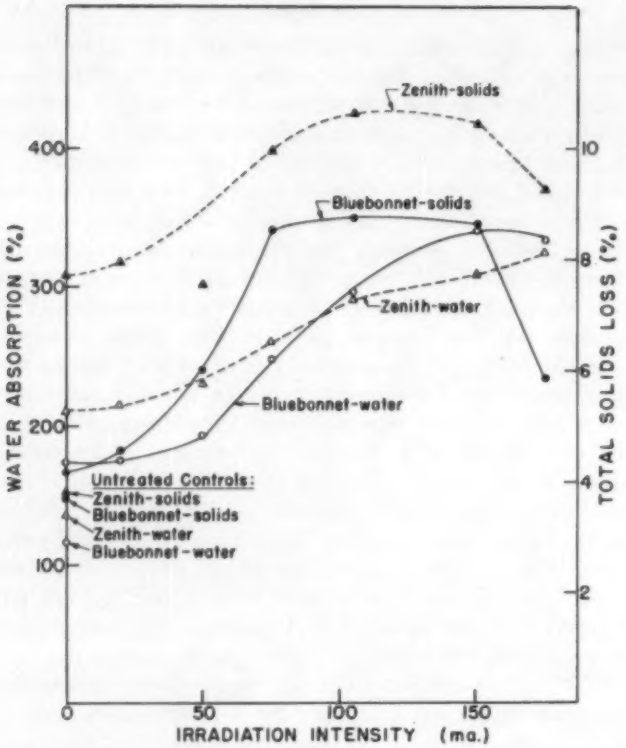

Fig. 2. Hydration characteristics of rice as influenced by irradiation for 5 minutes at 2 mm. of mercury.

maximum for Bluebonnet was reached at 150 ma. The change in amount of solids loss occurred at the same intensity as it did in the case of Zenith. Since treatment time and pressure were constant in this study, the effects of vacuum on hydration were constant regardless of current intensity. Thus the improvement in water absorption with progressively higher radiation doses was attributable to the effects of irradiation.

An intensity of 175 ma. at 2 mm. of mercury for 5 minutes was the highest level that could be used without browning the rice: at higher levels all of the rice became distinctly darker with a scattered occurrence of browned grains. However, using 15 g. of rice rather than 30 g. per treatment, it was possible to treat at intensities of about 210 ma. with less browning.

In many instances a scorched odor was noted after irradiation. Except when the odor was initially very pronounced, it gradually became less evident with storage. However, excluding those samples treated at 210 ma., no off-odor was detectable after they were hydrated under the described conditions.

Pressure. There was no great difference in hydration characteristics caused by varying pressure when rice was treated at 25 to 75 ma. for 5 minutes, in the pressure range of 2 to 8 mm. of mercury.

TABLE I

EFFECT OF TREATMENT PRESSURE AND CURRENT INTENSITY UPON THE HYDRATION CHARACTERISTICS OF RICE TREATED FOR 5 MINUTES

Variety	Irradiation Intensity	Water Absorption at Different mm. Pressures of Mercury			
		2	2.5	4	8
	ma	%	%	%	%
Bluebonnet	0	178	...	171	160
	25	176	...	173	194
	50	235	...	206	207
	75	248	...	224	217
Zenith	0[a,b]	...	119	103	72
	0[a]	...	121	114	110
	0	216	...	200	190
	25	215	...	202	204
	50	232	...	216	238
	75	256	...	239	239

[a] Water absorption determined 30 minutes at room temperature, 10 minutes at 70°C.
[b] Bleed located close to vacuum pump.

The vacuum pump capacity was, within certain limits, an important factor. The pressure increased slightly once irradiation was initiated, owing to increase in the gas temperature and in the increased out-gassing of the rice. A pump of still larger capacity minimized this, but even the expedient of employing a pumping capacity about 2.5 times greater did not correct this difficulty. The rate of gas or vapor evolution from the rice therefore must be a limiting factor. Thus, with a given pump, a Bluebonnet rice with 15.5% moisture required up to 3 minutes to reach 2 mm. of mercury pressure, whereas at 10.5% moisture, only 10 seconds were required.

The location of the pressure regulator, which in this case was a needle valve to admit air, was critical. When air was bled over the rice, using the set-up described in Fig. 1, higher water absorption values were obtained (Table I), especially at the highest pressure (8 mm. of mercury), than when air was admitted to the line connecting the tube to the vacuum pump, i.e., between No. 6 and No. 2, Fig. 1.

Time. At 50 ma. and 2 mm. of mercury pressure, any increase in

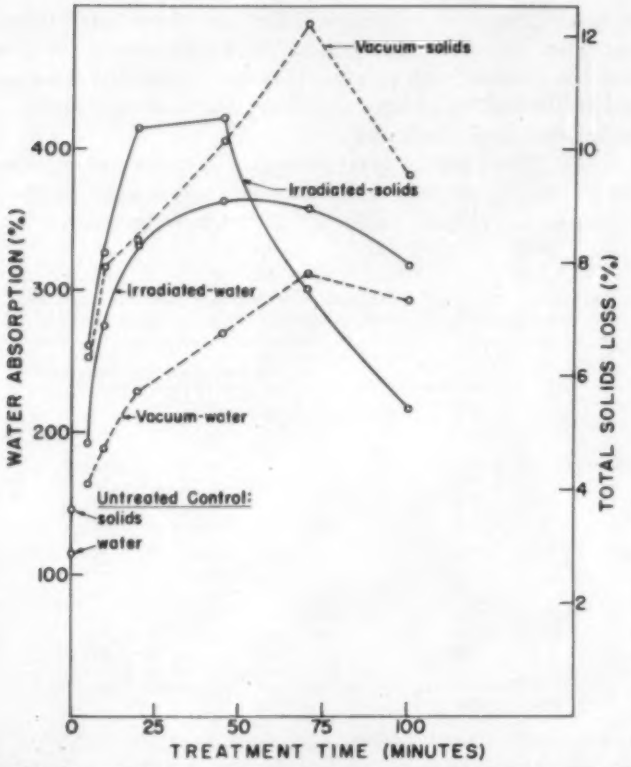

Fig. 3. Effect of duration of treatment upon the hydration characteristics of Bluebonnet variety rice (treated at 2 mm. of mercury and 50 ma. or 0 ma.).

treatment time over 45 minutes for Bluebonnet variety and 70 minutes for Zenith was ineffective in imparting higher water-absorption values (Figs. 3 and 4).

In both instances the next higher time level did not affect the amount of water absorption, but it did result in a great decrease in the amount of total solids loss to the treating water. This was also accompanied by a change in the texture, identical to the change noted in the intensity experiments previously described.

The effects of vacuum, *per se*, on both varieties apparently leveled off at about 70 minutes. However, at 45 minutes and above, vacuum played a more important part than irradiation in the increase in water absorption of irradiated Bluebonnet. This occurred because absorption in the irradiated samples did not increase as rapidly as in those

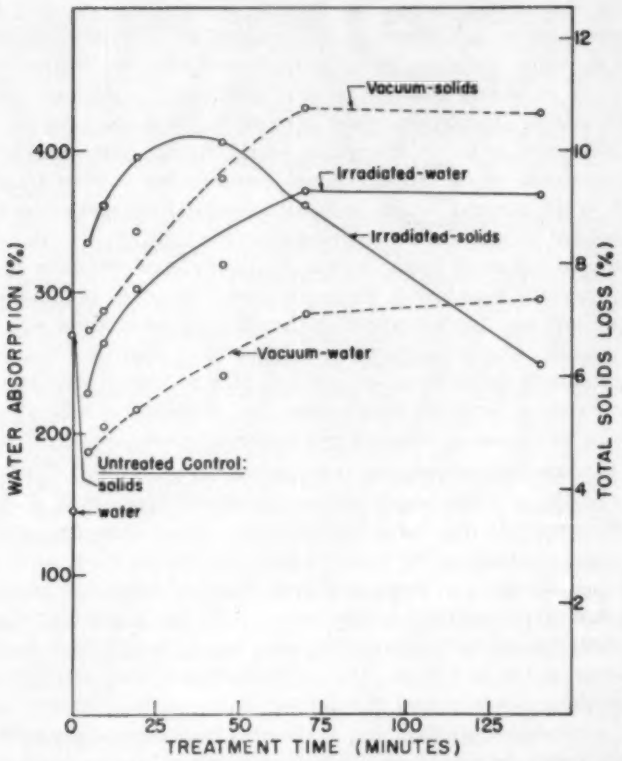

Fig. 4. Effect of duration of treatment upon the hydration characteristics of Zenith variety rice (treated at 2 mm. of mercury and 50 ma. or 0 ma.).

treated with only vacuum above 45 minutes.

Viscosity and Susceptibility to Beta-Amylase. Preliminary experiments (using a sample of Bluebonnet treated for 10 minutes at 2 mm. of mercury and at an intensity of 50 ma.) indicate that the starch, which constitutes about 90% of the dry weight of the rice, is not greatly changed by irradiation. This evidence was obtained from amylograph viscosity evaluations and tests for beta-amylase susceptibility, where there were no appreciable differences between the behavior of the treated rice and its control.

Discussion

Difficulty was experienced in reproducing exact water-absorption values under identical treatment conditions. A statistical analysis

showed that of the variability that occurred, 75% was due to the treatments *per se*, and the other 25% existed in the evaluation process. When the same operating variables were used with two different assemblies (i.e., these rigs differed in that different transformers, vacuum pumps, and irradiation chambers were used), there was a highly significant difference in water absorption of the treated samples. However, the magnitude of the rig-effect was constant for various treatments tested. While some difficulty occurred in reproducing absolute values, no problem existed in the reproduction of trends. It was possible to obtain peak values of water absorption for the same duration of irradiation when the experiment was replicated. The other points disclosed by the statistical analysis were: 1) the effect of irradiation was highly significantly more pronounced on Bluebonnet than on Zenith; and 2) the standard deviation of means of duplicate determinations (including variability for both replication and evaluation) was about 12. Thus for determining whether two treatments were different, a difference of twice the standard error of differences, or 34 ($= 2\,\sigma\,\bar{d} = 2\sqrt{2}\,\sigma\bar{x}$ $= 2\sqrt{2}\ 12$), was considered to be statistically significant.

When each of the operating variables – time, intensity, and pressure – was changed as the other two were kept constant, peak values for water absorption were produced. Consequently an assumption was made that when these optima were used in combination, a product with even greater water-holding capacity would result. Such an experiment run at 150 or 175 ma. for 25 minutes at 2 mm. of mercury did not produce this anticipated result on either variety. In fact, it gave lower water-holding capacities. Additional runs were made with Bluebonnet, holding 145 ma. and 2 mm. of mercury constant and varying treatment times up to 30 minutes: the maximum occurred at about 15 minutes.

Heat is generated in the glow discharge process. As a result, the temperature of the tube walls and the materials exposed to the discharge will increase to a point where the losses are equal to the input. This point will depend on the kind of gas, gas pressure, and current. Thus with the time held constant, greater amounts of heat are produced at the higher current intensities. With the latter constant, longer times permit the temperature to rise since such an equilibrium has not been attained. This may explain the decreased water absorption and total solids loss for times greater than 15 minutes at or above 145 ma., or for periods longer than 70 minutes at 50 ma. Possibly the absorption of heat by the rice at these time and current combinations could have partially sealed the surface of the rice grains and this could have retarded water absorption. This hypothesis is also useful

in explaining why there were decreased losses in total solids to the treating water, and why there were textural changes on the surface of the rice, i.e., changes in cohesiveness, under these conditions. For example, Bluebonnet treated for 15 minutes at 145 ma. and 2 mm. of mercury lost 12.7% solids to the cooking water during the hydration test, whereas 30 minutes under the same conditions gave a 5.7% loss when hydrated.

In these studies the use of a nonirradiated vacuum-treated rice represented only a poor approximation of a "true control." The major point of divergence was the temperature difference during treatment. In the case of the nonirradiated sample, the temperature gradually dropped owing to evaporative cooling; whereas during irradiation the temperature rose. Thus it cannot be discounted that the difference between these samples may have been due largely and possibly entirely to the effects of heat. These heat effects will be described in a subsequent paper, as will the results of cooking tests. Also the possible control of cohesiveness by the use of operating variables beyond those that produced maxima for water absorption will be included.

In summary, these studies have shown that milled rice, upon gas-plasma irradiation, becomes chalky, opaque, and extensively checked. The treatment resulted in a marked increase in the amount of water absorption and a greater swollen appearance of the irradiated rice, over that of the vacuum control, when they were immersed in hot water. Similar but much less extensive changes in appearance and hydration characteristics were evident in the nonirradiated vacuum-treated controls. In view of these changes, the use of treatment methods described should be studied to reveal their potential for the production of convenience rice products, i.e., products that cook faster or that have modified culinary properties.

Acknowledgments

The authors wish to thank Harold J. Deobald, SURDD, for his helpful suggestions and advice; Austin T. Harrell, Superintendent of the LSU Rice Experiment Station, Crowley, Louisiana, and members of his staff for supplying the rice and facilities for drying it. We are also sincerely grateful to E. Fred Schultz, Jr., Biometrical Services, SURDD, for his comprehensive statistical analyses.

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GAS PLASMA IRRADIATION OF RICE
II. Effect of Heat on Hydration and Cooking Characteristics[1]

J. T. Hogan and A. S. Roseman[2]

ABSTRACT

The hydration characteristics of rice are greatly changed by the simultaneous application of heat and reduced pressure to milled rice, i.e., by a heat-vacuum treatment, or by subjection to a gas plasma irradiation under comparable conditions of pressure, time of treatment, and temperature. The water absorption capacity is slightly greater for vacuum samples prepared at 45°C. and above than for samples irradiated at the same temperatures. Rices treated with vacuum, vacuum heat, or irradiation, cook faster and have different appearances than cooked untreated (raw) rice. A substantial reduction in cooking time of the treated materials is brought about by the use of a 15- to 20-minute presoak period.

In a previous paper (2) the authors reported that the gas plasma irradiation of milled rice increased its water absorption capacity so that the treated rice occupied a greater volume after a brief immersion in hot water than the nonirradiated vacuum-treated rice. Since temperatures attained during irradiation of the rice were higher than those

[1] Manuscript received February 13, 1961. Contribution from the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana.

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found for vacuum treatment without irradiation, the use of the latter as a control was questioned. Irradiation caused the temperature to rise, whereas the straight vacuum treatment caused the temperature to drop during exposure to the experimental conditions.

The object of the present investigations was primarily to ascertain whether the application of heat during preparation of the vacuum control could increase the water absorption capacity of the treated material to the same level as rice irradiated under similar conditions of time, pressure, and temperature. The secondary purpose was to evaluate the samples prepared in this study, as well as those previously processed (2), in terms of cooking time and characteristics of the cooked rice.

Materials and Methods

Rice. The milled Bluebonnet-50 and Zenith rices used in the experiments were prepared from the same lots of 1958 foundation seed stocks described in the previous paper (2).

Treatment Apparatus. The same equipment was used for treatment of the rice, except that the irradiation chamber originally described (2) was modified in an attempt to control the temperature. The chamber was enclosed by a concentric glass jacket, 70 mm. o.d. by 53 cm. long. This outer cylinder was attached by rubber stoppers to the inner one. By means of two glass sidearms on the outer cylinder, water or steam at the desired temperature was passed through the jacket. Saturated free-flowing steam at atmospheric pressure was used to maintain 100°C.

Irradiation. In both the irradiation as well as the nonirradiated vacuum treatments, temperature was controlled by passing water or steam through the jacket. A longer time was required to attain a pressure of 2 mm. of mercury when the irradiation chamber was heated. This was due to the faster rate of vapor loss caused by the initially higher temperatures. Preliminary experiments showed the longest period required was about 4.5 minutes. Therefore, in the modified apparatus, a constant time of 5 minutes was used in all runs to allow for evacuation of the system. After this interval, the timing of the treatment was initiated. Thus a nominal treatment time of 30 minutes required 5 minutes for pump-down time plus the 30 minutes specified.

Temperature Measurement. For estimation of the temperatures attained during any treatment, thermocouples were taped on the surface of the irradiation chamber. The external use of thermocouples was necessary because the placement of a thermometer or thermocouple

inside the treatment chamber during irradiation caused an abnormal glow discharge. Investigation showed that the jacket temperature varied from 1° to 4°C. above that of the rice as measured with a thermometer placed in the rice at the conclusion of a treatment.

Cooking Tests. Samples treated in this investigation and aliquots from those used for water uptake determinations (2) were appraised for cooking time and cooking characteristics. Rice (5 g.) was cooked in 60 ml. of distilled water over direct heat in a covered crystallizing dish 50 mm. o.d. by 70 mm. tall. Periodically, samples were withdrawn and pressed between glass slides, 2 by 3 in., to test for the presence of uncooked, white, hard centers. After they had disappeared the samples were subjectively checked to see if they were completely soft. The hard centers test had been suggested (1) as a method for cooking-time determination. When the samples were considered subjectively as done, the time was recorded and subsamples were given to a taste panel. The panel was instructed to rate the sample for doneness, flavor, odor, and appearance.

Using the same cooking method, larger-scale cooking-time appraisals were run on 60-g. quantities of rice in 350 ml. of water. This permitted comparison of cooking tests to doneness on a small scale versus those on a larger scale.

Results and Discussion

There was very little difference in amount of water uptake when determined by the previously established method (2), between rices irradiated for 30 minutes at 50 ma., 2 mm. of mercury, and the non-irradiated vacuum-heat treated samples. Although the data are not included here, the same parallelism existed between samples that had been similarly treated for 5 minutes. There was in general, a progressive increase in water uptake upon evaluation, when the treatment temperature was increased. The greatest single increase in water uptake occurred when the treatment temperature was raised from 95° to 100°C. The latter temperature was produced by free-flowing steam, whereas hot water was used for the former. Steam at 100°C. has a total heat capacity of about 1,150 Btu per lb., whereas water at 95°C. has only 161 Btu per lb. Thus steam is a much more efficient heating medium. This would explain the sharp increase in effect when the temperature was raised only 5°C. In addition, since at 95°C. the water contained in the rice is still in the liquid phase, the effect at this temperature is not as great as at 100° steam temperature. The expansion of water in the rice in going from water at 100°C. to steam at 100°C. is approximately 1,600-fold (specific volume of water at 100°C.

= 0.0167 cu. ft. per lb.; specific volume of steam at 100°C. = 26 cu. ft. per lb.). Thus, the tremendous expansion of steam could very well be an important factor responsible for the increased hydration properties induced in the product prepared at 100°C.

In earlier studies (2), a wide difference was shown in the hydration characteristics between the irradiated and the nonirradiated vacuum-treated samples. This difference was eliminated in the present study by application of heat during preparation of the vacuum control. This observation leads to the conclusion that at least the major effect of irradiation upon hydration is attributable to the heat produced by the gas plasma.

The initial point on Fig. 1 for the irradiated sample did not fall on the curve. Data for this point were obtained from rice treated in an unjacketed tube. Therefore, the temperature attained during treatment was entirely dependent upon heat produced during irradiation.

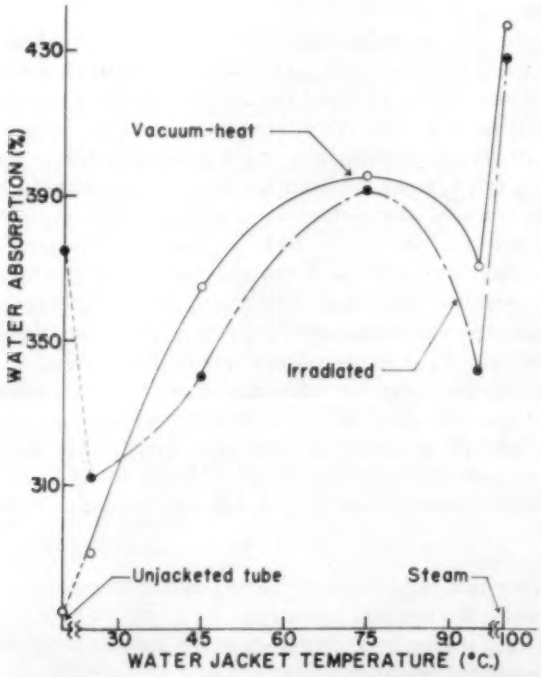

Fig. 1. Effect of temperature at time of irradiation (50 ma., 30 minutes, 2 mm. mercury), and vacuum treatment (0 ma., 30 minutes, 2 mm. mercury), upon the hydration characteristics of rice, Bluebonnet variety.

Thermocouples on the outside of the glass cylinder, immediately under the rice, indicated that 47°C. was the highest temperature attained after 30 minutes of treatment. Should this point on the curve be shifted laterally to 47°C., it would then fall in a much more logical place in relation to the rest of the curve.

An analogous situation existed for the first point on the curve for the nonirradiated sample. However, this point did not appear to be out of place. The temperature indicated by the thermocouples was about 23°C.; consequently it is almost correctly placed, as regards temperature, in its present position. The data from 5-minute treatment time samples confirmed this concept. Judging from the slope of the curve, in the 95° to 100°C. interval, it may be possible to get much greater changes in the amount of hydration by the use of temperatures above 100°C. Furthermore it may be assumed that more rapid and more efficient methods of heating, such as radiant or dielectric heating, could induce very high amounts of hydration when used for shorter times at lower temperatures.

The rapid loss of volatilized material that occurred during the treatments probably produced small fissures or channels for the escape of the volatilized material from the interior of the grains up to and through the surface. The resultant network could serve as ready pathways for more complete and rapid hydration when grains were immersed in water. Some credence for this theory comes from the following observations: the surfaces of all the treated rice grains were uniformly checked; the grains had lost their translucency and had become opaque; the surfaces of the cooked treated grains were very rough and irregular; and the cooked grains were greatly enlarged.

To accomplish the secondary objective of this study, cooking tests were run to determine if the times required for cooking to doneness were related to the hydration capacities shown by water-uptake determinations, Tables I and II. The samples which had high water absorption capacities failed to show any appreciable difference in cooking times as compared with those with the lower hydratabilities. Additional observations made on the cooking methods and results are as follows:

1. Cooking in a large excess of water resulted in excessive breakage, especially when the rice was presoaked.
2. The hard centers test did not show any consistent relationship to subjective completeness in cooking.
3. Cooking tests on 5-g. quantities of rice generally required about the same cooking time as those done on 60-g. amounts.

TABLE I

COOKING TIME OF RICE SAMPLES TREATED FOR 5 MINUTES AT 2 MM. MERCURY, AND AT VARIOUS LEVELS OF CURRENT INTENSITIES

Irradiation Intensity	Cooking Times at 100° C.							
	Bluebonnet				Zenith			
	20-Minute PS[a]		No PS		20-Minute PS		No PS	
	No HC[b]	Done	No HC	Done	No HC	Done	No HC	Done
ma	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
Untreated	17.5	20.0	20.0	20.0	12.0	14.2	17.0	24.0
0	1.7	9.2	18.0	18.0	4.7	7.7	18.0	24.0
25	1.7	9.5	19.0	19.0	5.0	7.7	18.0	23.2
50	1.0	6.5	18.5	18.5	2.0	8.0	16.7	20.7
75	2.2	7.2	19.0	19.0	2.0	7.7	16.0	20.0
110	2.7	7.0	16.5	16.5	2.2	7.0	13.0	18.0
145	1.2	8.0	17.7	17.7	...	...	....	....
150	3.5	...	15.7	....	4.0	9.0	12.0	18.0
175	3.7	6.7	16.5	16.5	8.0	8.5	13.0	19.0
210	3.2	8.0	....	....	6.0	9.0	12.2	18.0

[a] PS = presoaking.
[b] HC = hard centers.

4. The benefits of the treatments (vacuum, vacuum-heat, or irradiation) were more evident in cooking-time determinations, when a presoak was used. Limited observations indicate that 15 minutes was the minimum presoak time required.

5. At higher irradiation intensities where browning was produced and/or where rice was off-color, off-flavors and off-odors were detectable in the cooked samples.

TABLE II

COOKING TIME AFTER 20 MINUTES' PRESOAK OF BLUEBONNET RICE IRRADIATED FOR 5 OR 30 MINUTES AT 50 MA. INTENSITY AND AT 2 MM. MERCURY; AND OF NONIRRADIATED VACUUM-TREATED CONTROLS PREPARED AT VARIOUS TEMPERATURES (5 or 30 Minutes at 0 ma., 2 mm. Mercury)

Treatment Temperature	Cooking Times at 100° C.							
	Treated 5 Minutes				Treated 30 Minutes			
	Nonirradiated		Irradiated		Nonirradiated		Irradiated	
	No HC[a]	Done	No HC	Done	No HC	Done	No HC	Done
°C	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*	*minutes*
Conv.[b]	3.25	7.25	3.0	8.0	...	...	...	...
Conv.	....	...	...	...	4.5	8.0	8.5	8.5
25	2.7	8.5	3.5	8.5	5.0	9.0	5.5	9.0
45	3.5	6.0	6.0	8.2	3.5	7.5	7.7	7.7
75	3.0	9.0	4.0	8.5	4.0	8.0	3.5	7.0
95	3.2	8.2	2.5	7.2	3.7	7.0	3.5	8.5
100[c]	2.7	8.0	5.0	9.0	1.7	6.2	4.2	9.7

[a] HC = hard centers.
[b] Conventional treatment apparatus.
[c] Heated by steam rather than by water.

Acknowledgments

The authors wish to express their appreciation to Russell B. Stone, Jr., and J. C. Webb, Agricultural Engineering Research Division, USDA, Knoxville, Tenn., for their assistance with the equipment and for the preparation of some of the samples; and to Austin C. F. Mason, Glassblower, Southern Utilization Research and Development Division, for construction of experimental irradiation chambers and loading devices, which were extensively used in an earlier study and modified for the present investigation.

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ACCESSIBLE SULFHYDRYL GROUPS IN DOUGH[1]

W. BUSHUK

ABSTRACT

The rapid reaction of iodate ions with sulfhydryl groups was applied to the determination of the accessibility of these groups in flour-water doughs. The maximum amount of iodate that reacts in a dough was obtained graphically, from amperometric titrations of residual iodate, by plotting residual iodate vs. iodate added and extrapolating to zero residual iodate. Reasonable agreement with calculated values for the number of sulfhydryl groups was obtained for doughs which contained added glutathione or thiolated gelatin, and no reaction with iodate was obtained when sulfhydryl groups were blocked with N-ethylmaleimide. Increases in accessible sulfhydryl groups were obtained for doughs prepared from flours of decreasing particle size, doughs subjected to prolonged mixing, and doughs treated with guanidine hydrochloride to break hydrogen bonds. These results indicate that iodate ions react primarily with accessible sulfhydryl groups. Illustrative results for doughs from eight different flours ranged from 4.0 to 7.5 μeq. per g. of protein. Within a single class of flour, the number of accessible sulfhydryl groups increased with increasing protein content. The method was also used to determine the number of accessible sulfhydryl groups in flour-water slurries for one sample of flour.

Recent studies have shown that sulfhydryl groups of flour proteins play a major role in determining the mixing properties of dough (16). In addition, it now seems certain that oxidative improvement of flour quality involves the sulfhydryl group (5,10, and papers cited therein). Current studies in this and the Western Regional Laboratories also

[1]Manuscript received February 10, 1961. Paper No. 203 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg 2, Canada.

suggest that it is the sulfhydryl groups that are initially accessible, or subsequently made accessible by prolonged mixing, that are oxidized (4,21). Accordingly, a method for the determination of the *accessible* sulfhydryl groups in dough would probably be more useful from a practical viewpoint than current methods that measure the *total* sulfhydryl content of flour or of various protein fractions obtained from it (2,8,14,15,17–20). References by Mecham, Pence, and coworkers (14,20) which deal with the application of an amperometric titration method to the determination of total number of sulfhydryl groups in flour slurries are most pertinent to the present study.

This paper describes a method for the determination of accessible sulfhydryl groups in dough. The method, based on the rapid reaction of protein sulfhydryl groups with iodate ions, is an extension of the observation made in this Laboratory several years ago that flours of different type react with characteristic amounts of iodate (9). Usefulness of the method is demonstrated by a study of some of the important factors that control the accessibility of sulfhydryl groups and by a determination of the accessible sulfhydryl contents of doughs from eight different flours and of water-flour slurries from one flour.

Materials and Methods

The apparatus, reagents, and methods used for preparing the dough, and extracting and measuring residual iodate, are the same as used in earlier studies of the bromate reaction in dough (3,6,7); however, the procedure will be described again to facilitate the use of the method without referring to several other papers.

Apparatus. (a) The dough mixer must be adapted for mixing under nitrogen; the GRL mixer (12) is used in this Laboratory. (b) Magnetic stirrer with an external speed-control rheostat. (c) Polarizing and current-detection unit which may be constructed according to the circuit diagram given by Kolthoff and Lingane (13). In the unit used, a double-throw, double-pole switch was installed so that the galvanometer could be used for measuring both positive and negative currents. The galvanometer used has a sensitivity of 0.1 μamp. per unit scale division (100 scale divisions = 60 mm.). (d) The indicator is a platinum electrode (Beckman 39271) and the reference is a calomel electrode (Beckman 39270). The platinum electrode was cut down to the same size as the calomel electrode and both are mounted on a holder about 15 mm. apart.

Reagents. (a) Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), 155.1 g. per liter. (b) Sodium hydroxide, 26 g. per liter. (c) Sodium thiosulfate, 0.001*N*. (d) Potassium iodate, 0.001*N*. (e) Potassium iodide, 30%. (f) Sulfuric

acid, 10%. (g) n-Octyl alcohol.

Method. The method involves determination of residual iodate in flour-water doughs immediately after mixing. The analysis is repeated with replicate doughs containing increasing amounts of iodate until three or four points representing doughs containing residual iodate are obtained. The maximum amount of iodate that reacts in the dough prepared under a definite set of conditions from a given flour is determined by plotting residual iodate against iodate added and extrapolating to zero residual iodate.

The dough is prepared from 100 g. (or less if a suitable mixer is available) of flour by mixing under nitrogen for 5 minutes at a practical absorption (60% was used in the present study except where indicated otherwise). In the standard procedure, the iodate is added at the beginning of the 5-minute mixing. The flour should be stored overnight under nitrogen to remove oxygen, which competes with iodate for the sulfhydryl group. Oxygen is removed from the liquids used to mix the dough by bubbling nitrogen through for 5 minutes.

Residual iodate is extracted from a 20-g. portion of the dough by dispersing in a Waring Blendor for 3 minutes at low speed with 90 ml. of water, 12.5 ml. of zinc sulfate solution, and 12.5 ml. of NaOH solution; 5 drops of octyl alcohol are added to prevent excessive foaming. Two 50-ml. portions of the dispersion are centrifuged for 10 minutes at 1,500 $\times$ *g*, and duplicate 20-ml. aliquots of the supernatant are titrated.

The titration medium comprises 20 ml. of supernatant, 5 ml. of 0.001*N* thiosulfate, 5 ml. of 10% sulfuric acid, and 3 ml. 30% potassium iodide. Excess thiosulfate is titrated with 0.001*N* potassium iodate, and the end point is established amperometrically. A reagent blank is run daily on 5 ml. of 0.001*N* sodium thiosulfate. The titration medium is stirred with a magnetic stirrer throughout the titration.

The polarizing unit was checked in the usual manner. Polarograms of Fig. 1, A, show that with the electrodes used the amperometric titration can be conveniently made at 0 volts applied potential. Figure 1, B, shows that at this potential the relationship between the current and the volume of titrant is linear. The sensitivity of the electrode used is about 39 μamp. per ml. of 0.001*N* iodate.

The maximum amount of iodate that reacts in a particular dough is determined as follows: Residual iodate is first determined for a series of replicate doughs containing increasing increments of potassium iodate. The residual iodate (expressed in ml. of titrant) is then plotted against iodate added (in p.p.m. of flour) and the maximum amount that reacts, or the iodate saturation value (ISV), is obtained

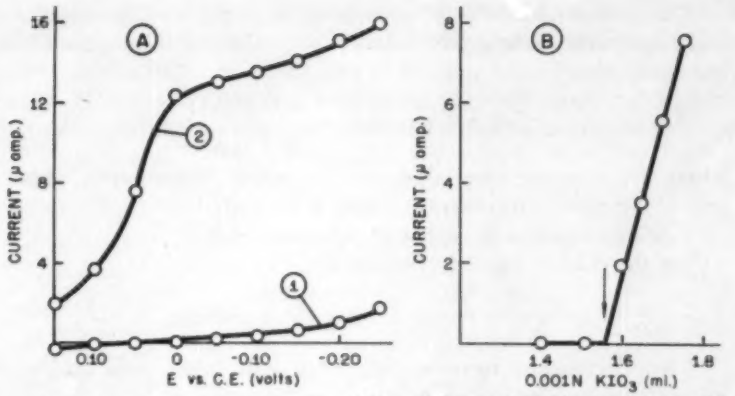

Fig. 1. A, current-potential curves at the platinum electrode for the titrating medium comprising 20 ml. of water, 2 ml. of 0.001*N* thiosulfate solution, 5 ml. of 10% sulfuric acid, and 3 ml. of 30% potassium iodide solution. 1, residual current; 2, 0.36 ml. of 0.001*N* KIO_3 in excess of the thiosulfate. B, current-volume of titrant curve at 0 volts applied potential.

by extrapolation. Typical results for a dough from a straight-grade flour of 13.2% protein content milled from hard red spring wheat are shown in Fig. 2. The ascending limb of the curve is linear, so that a good extrapolation can be made with three or four points. Since the ascending limb will have a slope of one (if the same ordinate and abscissa units are used), a reasonably good estimate of the ISV can be obtained from a single determination at an iodate level higher than ISV.

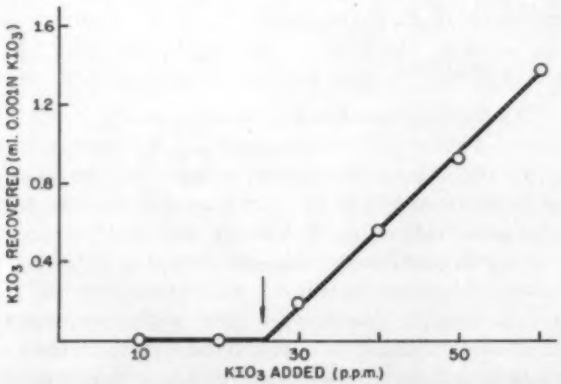

Fig. 2. Iodate recovered vs. iodate added curve for doughs from a straight-grade flour of 13.2% protein mixed in nitrogen for 5 minutes. Iodate saturation value (indicated by arrow) is 25.5 p.p.m.

The accessible sulfhydryl content in μeq. per g. of protein, of a dough prepared under a particular set of conditions, is calculated from the equation:

$$\mu\text{eq. SH/g protein} = \frac{100\ \text{ISV}}{\text{P} \times 35.67}$$

where ISV = iodate saturation value in p.p.m. of flour, 14% m.b.;
P = protein content %, 14% m.b.; and
35.67 = equivalent weight of potassium iodate.

For the data of Fig. 2 this equation gives:

$$\frac{100(25.5)}{13.2 \times 35.67} = 5.4\ \mu\text{eq. SH/g protein}$$

This calculation assumes that iodate ions react with sulfhydryl groups according to the reaction

$$6\text{RSH} + \text{IO}_3^- \rightarrow 3\text{R} - \text{S} - \text{S} - \text{R} + \text{I}^- + 3\text{H}_2\text{O},$$

in which the molar ratio of sulfhydryl groups oxidized to iodate at the equivalence point is 6.

The reproducibility of the method was checked by a series of determinations on doughs from the same flour. Standard error obtained was about ± 0.1 μeq. per g. of protein.

When iodate is added to a dough, the amount equal to the ISV reacts very rapidly; however, if a long reaction time is allowed there is a further progressive reaction of iodate. This seems to involve the less accessible sulfhydryl groups reached by diffusion of iodate into the hydrated flour particles. The rate of this reaction is constant for a wide range of iodate concentrations and is equal to 0.04 p.p.m. of potassium iodate per minute or 0.2 p.p.m. for the 5 minutes of mixing used in the standard procedure. The error introduced by this secondary reaction of iodate is generally less than 1% and is not considered as serious.

Results and Discussion

Specificity. Two types of experiments were made to check the specificity of the iodate reaction in dough. In the first type, the sulfhydryl groups were blocked by incorporating into the dough excess N-ethylmaleimide, mixing for 5 minutes, and then adding the iodate and mixing for an additional 5 minutes. Complete recoveries of iodate were obtained at potassium iodate concentration of 10, 15, and 20 p.p.m.; and the straight line through these points extrapolated to zero ISV. Accordingly, under the specified conditions, iodate does not react in significant amount with any group that is not blocked by N-ethylmaleimide.

In the second type of specificity experiment, the sulfhydryl content

of the dough was increased by additions of glutathione (reduced) and of thiolated gelatin (Schwartz, Thiogel A; molecular weight = 10,000, –SH content 0.93 per mole). Since, for this experiment, the actual source of sulfhydryl groups is immaterial, the –SH compound and the iodate were added to the flour at the same time. The results obtained are given below.

		SH Content	
Glutathione	*ISV*	*Experimental*	*Calculated*
mg/100g flour	*ppm*	*μeq/g protein*	*μeq/g protein*
0	25.5	5.4	...
12	40.5	8.5	8.4
24	58.0	12.2	11.3
Thiolated Gelatin			
375	41.7	8.7[a]	7.8[b]
750	59.5	12.0[a]	10.1[b]

[a] The amount of protein added was included in this calculation.
[b] Calculated on the basis of 0.93 —SH groups per 10,000 molecular weight and total protein content of 13.2% + added thiolated gelatin, i.e. 13.58 and 13.95%.

The agreement between the experimental and the calculated values for the sulfhydryl contents given above is reasonably satisfactory. Two reasons can be given to account for the higher experimental values. First is that addition of sulfhydryl compounds to dough might increase the number of accessible sulfhydryl groups in the dough. This does not seem unlikely for glutathione, which has a marked effect on the handling properties of dough; however, it may not be the case with thiolated gelatin, which does not affect the handling properties of dough under the conditions used. On the other hand, it might be that the molar equivalence ratio of sulfhydryl groups to iodate of 6 is too high. Lower ratios would yield lower experimental values. A lower molar ratio would imply that sulfhydryl groups are oxidized by iodate to a state higher than that of the disulfide group. Recent studies by Hird and Yates (11) on solutions of reduced gluten indicate that the molar ratio for sulfhydryl groups oxidized to iodate added is about 4 and depends on the relative concentration of sulfhydryl groups to iodate. Further study is necessary to determine if this ratio applies also to doughs. If this is found true, then the values of accessible sulfhydryl content given in this paper would have to be decreased by one-third; however, the relative positions of the various doughs studied would remain unchanged.

Physical Accessibility. Four types of experimental doughs were used to check the validity of the presumption that the rapid reaction of iodate is with the physically accessible sulfhydryl groups: doughs prepared from flours of different particle size; doughs that were subjected

to prolonged mixing under nitrogen; doughs of different absorption mixed for 5 minutes; and doughs treated with guanidine hydrochloride (hydrogen bond-breaking reagent). Results for each type of dough will be discussed separately.

The flours of different particle size were milled in the Laboratory from hard red spring wheat. A coarse farina was milled first and portions of it were reduced completely to a finer farina and flour. A fourth sample was made by ball-milling a portion of the flour. The accessible sulfhydryl contents of the doughs from these flours, together with the description of the flours, are given below.

Flour	*Particle Size*	*Protein Content*	*Sulfhydryl Content*
		%, 14% mb	*μeq/g protein*
Coarse farina	thru 30GG, over 72GG	11.7	4.8
Farina	thru 72GG, over 10XX	11.6	5.3
Flour	thru 10XX	11.6	5.7
Ball-milled flour	....	11.6	6.6

The above results are consistent with the hypotheses that the method determines accessible sulfhydryl groups and that the number of them increases with decreasing flour particle size, i.e., with increasing specific surface area. The increase in sulfhydryl content is comparable to the increase in the rate of bromate reaction in doughs from similar flours (4). This type of accessibility, depending on particle size, is different from the molecular accessibility encountered in proteins in solution. From the practical viewpoint, these results suggest that the effects of milling properties of wheat and milling technique on bromate requirement and response of the resulting flour depend on the number of sulfhydryl groups made accessible during the milling process.

The effect of prolonged mixing under nitrogen on the sulfhydryl contents of doughs of 50 and 70% absorption is shown by results given below. In these experiments, iodate was present in the dough during the last 5 minutes of mixing.

Mixing Time	*ISV*		*Sulfhydryl Content*	
	50%	70%	50%	70%
minutes	*ppm*	*ppm*	*μeq/g protein*	*μeq/g protein*
5	27.0	23.0	5.7	4.9
10	42.0	28.5	8.9	6.6
15	51.0	35.0	10.8	7.4

Prolonged mixing produced a definite increase in the number of sulfhydryl groups that are accessible to iodate. The increase produced is higher for the dough of lower absorption. Thus, mixing time and

absorption condition the accessibility of sulfhydryl groups and thus the response to improvers.

Another type of accessibility was observed in doughs treated with guanidine hydrochloride, a commonly used hydrogen bond-breaking reagent. Figure 3 shows the results that were obtained for doughs

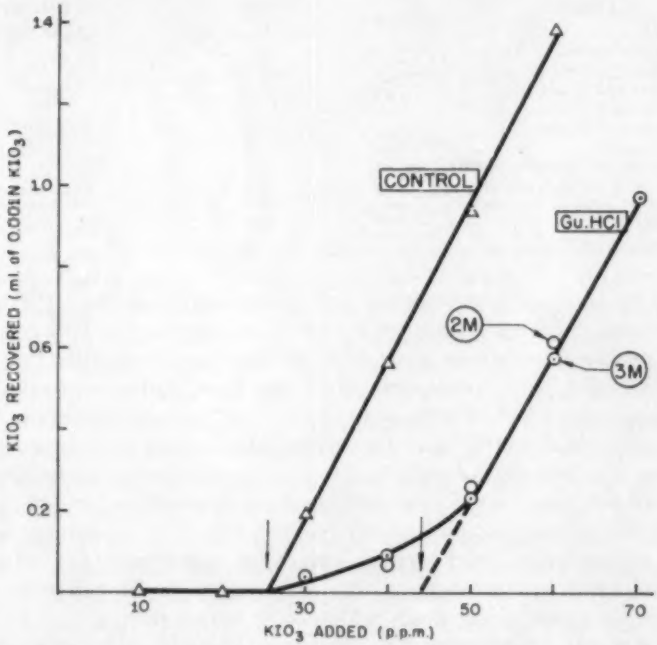

Fig. 3. Iodate recovered vs. iodate added curve for control doughs and for doughs treated with two concentrations of guanidine hydrochloride.

treated with 2*M* and 3*M* guanidine hydrochloride (liquid basis). It seems that the number of sulfhydryl groups reacting depends on the amount of iodate that is added to the dough; but a limit is reached when excess iodate is present. Results were the same for the two concentrations of guanidine hydrochloride used. The sulfhydryl content of the treated doughs obtained by extrapolating the straight portion of the curve (dashed line in Fig. 3) is 9.4 μeq. per g. of protein. Whether this actually represents the total sulfhydryl content of the dough would require a more intensive study.

Accessible Sulfhydryl Groups in Doughs from Various Flours. The method described in this paper was used to obtain data illustrating

differences in the accessible sulfhydryl contents of doughs prepared from flours of different type and quality. Results given below were obtained for doughs of 60% absorption mixed in nitrogen for 5 minutes at 30°C.

Flour	*Protein Content* %, 14% mb	*Sulfhydryl Content* μeq/g protein
Hard red spring		
Baker's special	12.5	4.0
Straight grade	13.2	5.4
Straight grade	13.3	5.4
Baker's strong	15.4	6.7
Garnet	11.4	7.4
Hard red winter (air-classified)[a]		
Low-protein fraction	10.8	4.2
High-protein fraction	20.4	7.2
Durum	12.9	7.5

[a] Supplied by The Pillsbury Co.

The above results show up two interesting features that deserve comment. Sulfhydryl contents for the first four hard red spring and the two hard red winter flours indicate that accessible sulfhydryl content depends on the protein content of the flour. These results provide a fundamental basis for the observation that bromate requirement for optimum loaf volume for high-grade flours seems to increase with increase in protein content (1). By contrast, an attempt to establish a correlation between the total sulfhydryl content and response to oxidation did not appear particularly fruitful (22). The second feature is the high accessible sulfhydryl content of the poor-quality Garnet flour of relatively low protein content, compared with the values for the other hard red spring flours which were milled from a high-quality bread wheat. More comprehensive studies of a wide range of varieties may well show that *accessible* sulfhydryl content of dough plays a significant role in determining baking quality.

Application of the Iodate Method to Flour Slurries. To provide a more direct basis for comparison between results by the iodate procedure and published results, the method described in this paper was used to determine accessible sulfhydryl contents of one flour in slurries of much higher water:flour ratios than that of dough. The experimental procedure used with flour slurries is as follows: The flour is blended with 95 ml. water in a Waring Blendor running at slow speed; 5 ml. of 0.0056*N* potassium iodate are added and given a 5-minute reaction time with the blender running, and finally the zinc sulfate and sodium hydroxide are added and blending continued for additional 2.5 minutes. The slurry is centrifuged and 20-ml. aliquots are

titrated for residual iodate. Usual precautions are taken to eliminate atmospheric oxygen during all operations.

The results that were obtained for a straight-grade flour of 13.2% protein for five dispersion times and four water:flour ratios are given below.

Total Dispersion Time	*Sulfhydryl Content*			
	50:1[a]	20:1[a]	10:1[a]	5:1[a]
minutes	*μeq/g protein*	*μeq/g protein*	*μeq/g protein*	*μeq/g protein*
7.5	10.4	7.2	5.6	4.5
12.5	10.4	7.6	5.6	4.7
17.5	10.2	7.6	6.1	5.0
27.5	...	8.0	6.7	5.4
37.5	...	8.0	7.4	5.6

[a] Water:flour ratio.

For all water:flour ratios, the number of accessible sulfhydryl groups increased with increasing dispersion time. This effect is analogous to the effect of prolonged mixing on the accessibility of sulfhydryl groups in dough (see above). The effect of prolonged mixing (dispersion) time decreased as the water:flour ratio increased. Although for the lowest water:flour ratio used, the accessible sulfhydryl content is slightly less than that obtained for a dough from the same flour by the standard procedure, the higher water:flour ratios gave considerably higher results. The results for the most dilute slurry indicate that a saturation point is reached in the accessible sulfhydryl content (10.4 μeq. per g. of protein) after 7.5 minutes' dispersion time. This value is slightly higher than the value of 8.1 μeq. per g. of protein obtained by Sokol, Mecham, and Pence (20) by amperometric titration of flour dispersions with silver nitrate. The present results indicate that accessibility of sulfhydryl groups to iodate ions in water-flour slurries increases with increasing dispersion time and water:flour ratio. Accordingly, if the iodate procedure is used to estimate the relative sulfhydryl content of a number of flours, the same conditions must be used for all samples.

Acknowledgment

The author wishes to express grateful acknowledgment to F. J. R. Hird, University of Melbourne, who put unpublished material at his disposal, and to R. A. McLeod for technical assistance.

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COMPLEXES OF AMYLOSE WITH SURFACTANTS[1]

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ABSTRACT

Eighteen surfactants, fatty acids and their esters with one, two, or more hydrocarbon chains, formed complexes with amylose having the same cell dimensions, as indicated by X-ray diffraction patterns. All surfactants used, with the exception of the diglycerides of hydrogenated soybean oil, greatly reduced the iodine affinity of amylose, but in no case was it reduced to zero. Soybean oil itself showed no indication of complex formation with amylose. For those surfactants containing a single hydrocarbon chain, the percentage of additive calculated for the hypothetical reduction of iodine affinity to zero, indicative of complete complex formation, appeared to be directly related to the percentage of the hydrocarbon portion of the molecule.

The mechanism by which certain surfactants retard some of the changes, particularly the increase in firmness, associated with the staling of bread is not completely understood. The ability of one such surfactant, polyoxyethylene monostearate, to complex with the amylose fraction of starch has been demonstrated quite conclusively (9,21) by its interference with the affinity of the amylose for iodine. Indications that monoglycerides (25) and sucrose monostearate (5) have similar effects have been reported. That the formation of such complexes of amylose in bread is the cause of the bread-softening action of these compounds has been postulated (9). However, surfactants of different chemical structures have been reported (11) to differ widely in their effects on crumb firmness. They likewise have been found (12) to cause different effects on curves obtained with the Brabender Amylograph for corn starch slurries containing soybean oil. Both the temperature at which viscosity increased during heating and the shape of the cooling curve were affected. These differences appeared to be related to the lengths of the hydrocarbon chains and the number of hydrocarbon chains in the molecules, as well as to the structures of the hydrophilic moieties.

The present study was undertaken to determine whether or not these reported differences between surfactants in respect to their effects on amylograph curves and crumb softness might be caused by differences in their ability to form complexes with amylose or differences in the dimensions of the complexes formed.

One method of measuring the extent to which amylose has under-

[1] Manuscript received October 31, 1960. Contribution from the Department of Home Economics, University of Illinois, Urbana, Ill.

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gone complex formation with compounds of this type is measurement of the reduction in its iodine affinity. Lord (9) and Schoch (21) reported that a linear relationship existed between the percentage of polyoxyethylene stearate or fatty acid added to starch or amylose and its iodine affinity. It was likewise shown that, on a weight basis, fatty acid was more effective in reducing iodine affinity than was this ester. The difference was attributed to difference in the relative sizes of the two molecules. An explanation proposed for the effect of surfactants or fatty acids on iodine affinity rests on the structure of the complexes involved. Hanes (6), in 1937, postulated a helical configuration for the starch-iodine complex, a hypothesis supported by later studies of Rundle and his co-workers (14,15,17,18,19,24). The latter suggested that the iodine molecules are oriented in a linear arrangement enclosed within and parallel to the axis of an amylose helix. X-ray diffraction studies led Mikus, Hixon, and Rundle (10) to propose a similar structure for complexes of fatty acids with amylose, with fully extended fatty acid molecules forming the core of the helix. Measurements of iodine-binding capacities of complexes containing palmitic, oleic, and lauric acids indicated that the greater the length of the fully extended fatty acid, the more amylose it bound. No similar comparison of the relative effectiveness of surface-active agents of a variety of structures has been made. In fact, the only reports in the literature on the effects of surface-active agents on iodine affinity appear to concern polyoxyethylene monostearate (9,21), monoglycerides (25), and sucrose monostearate (5), all of which have only one hydrocarbon chain.

X-ray diffraction studies of complexes of amylose and surface-active agents have not been reported, but complexes of palmitic, stearic, and oleic acids with amylose have been found to give X-ray patterns with nearly identical spacings (10), very similar to those for the amylose-iodine complex. However, Bear (3) concluded, from patterns obtained with amylose complexes of linear and branched alcohols, that the amylose helix can be enlarged to accommodate the branched-chain alcohols.

In the present study comparison was made of the efficacies of various surface-active agents in reducing the iodine affinity of amylose. X-ray diffraction patterns of purified complexes of amylose and the various surface-active agents were also compared.

Material and Methods

Materials. Amylose was prepared from commercial corn starch by selective precipitation with Pentasol, according to the method described by Lansky, Kooi, and Schoch (8). It was purified by two recrystallizations from water saturated with 1-butanol. After extraction in a

Soxhlet apparatus for 24 hours with 95% ethanol, the amylose was dried 4 hours at 50°C. under vacuum, and pulverized. The iodine affinity was 15.2%, a value low for pure amylose but indicating a sufficiently high percentage of the linear component for the present study.

Purified Complexes of Amylose with Surfactants. To a suspension of 0.300 g. corn amylose in 5 ml. water, 10.0 ml. of 1*N* potassium hydroxide were added, and the sample was held in a refrigerator with occasional stirring for 30 minutes, or until a clear solution was obtained. The solution was then neutralized to methyl orange with 0.5*N* hydrochloric acid and transferred into a 150-ml., three-necked round-bottom flask provided with a condenser and an efficient stirrer. The flask was placed in a water bath at 65°C. To it was added 0.100 to 0.150 g. of surface-active agent. Water was added until the volume of the mixture was approximately 60 ml., and it was stirred at 65°C. for 3 hours. The heating and stirring were then discontinued and the flask was left in the water bath overnight to cool slowly to room temperature. The precipitate was separated by centrifugation for 30 minutes in a Servall Superspeed Angle Centrifuge at 13,800 r.p.m. (about 24,300 × *g*). The precipitate was washed twice with hot carbon tetrachloride, dried for 1.5 to 2 hours at 65°C. under vacuum, powdered, and stored in a desiccator over sulfuric acid.

An aliquot of the sample was extracted exhaustively in a micro-Soxhlet apparatus with carbon tetrachloride. A second aliquot was extracted exhaustively with absolute methanol.

Infrared absorption spectra were obtained on all extracted samples. The Nujol mull technique was used.

Iodine affinity of samples which had been extracted with methanol was measured by the standard procedure of Schoch (22). The same procedure was used for the samples which had been extracted with carbon tetrachloride, except that the potassium hydroxide was neutralized with hydrochloric acid before the sample was added to it. In this way possibility of saponification of the complexed surfactant was eliminated; dispersion appeared to be as complete as in potassium hydroxide. In neither potassium hydroxide nor the neutralized solution was the complex dissolved, but a fine dispersion was obtained.

X-ray diagrams of samples after extraction with carbon tetrachloride or methanol were made with CuK_α radiation, nickel-filtered, and a cylindrical powder camera of 7-cm. radius. The powder samples were sealed in thin-walled glass capillaries of 0.7-mm. diameter. Exposure time was 4.5 hours at 15 ma. and 40 kv.

Effect of Surfactants on Iodine Affinity of Amylose. A sample of

amylose (37.8 mg., d.b.) was weighed into a 125-ml. Erlenmeyer flask. Approximately 1 ml. of water was added to suspend the sample. Five milliliters of 1*N* potassium hydroxide were added and the sample was immediately dispersed by stirring with a glass rod. It was then held, with occasional stirring, in the refrigerator for 30 minutes. The resulting clear solution was neutralized to methyl orange with 0.5*N* hydrochloric acid. A weighed amount of surfactant was added. Water (25 to 50 ml.) was added and the mixture was stirred in a constant-temperature water bath for 3 to 6 hours. Time and temperature of heating were varied with each surfactant to determine whether or not equilibrium had been reached. The solution was slowly cooled, without agitation, to room temperature and transferred quantitatively to a tared 250-ml. beaker. Ten milliliters of 0.5*N* potassium iodide solution were added. Sufficient water was added to bring the weight of the mixture to 100.9 g.

The potentiometric iodine titration was carried out by the method of Bates, French, and Rundle (1), as modified by Schoch (22), except that readings of the e.m.f. values were taken after intervals of only 1 minute following addition of each milliliter of iodine. Iodine affinity was calculated in the usual way.

Results and Discussion

Interaction between the amylose and the surfactants was indicated by the formation of precipitates in the cooled reaction mixtures after these substances had been heated together in water. Such precipitation of amylose has previously been brought about by addition of fatty acids (23), polyoxyethylene monostearate (5,9), sucrose monostearate, sucrose distearate, and polyoxyethylene sorbitan (5), as well as with a variety of other polar organic compounds. Bourne, Tiffin, and Weigel (5), estimating the amount of precipitation from turbidity measurements, observed that the amount of precipitate was proportional to the amount of surfactant up to a certain point beyond which additional surfactant had no effect. In the present study the amount of surfactant used provided a large excess over that needed to reduce the iodine affinity to a minimum value (cf. Table IV). However, with diglycerides prepared from hydrogenated soybean oil even such a large excess produced only a very little precipitate, although more than appeared possible from impurities in the diglyceride. Soybean oil itself yielded no precipitate with the amylose.

Exhaustive extraction with carbon tetrachloride removed only unbound surfactant from the precipitate, whereas methanol removed the bound surfactant as well. Although the weights of surfactants

extracted were determined only roughly after evaporation of the extracts, it was observed that the methanol extracts yielded larger residues than the corresponding carbon tetrachloride extracts in all cases, except with the precipitate from the diglycerides. That the surfactant remained bound after carbon tetrachloride extraction but not after methanol extraction was shown by the infrared spectra of the extracted products. Those extracted with carbon tetrachloride all showed a characteristic absorption maximum in the region of 1,725 $cm.^{-1}$, indicating the presence of carboxyl groups. Those samples extracted with methanol failed to show such a maximum. Further evidence that the surfactant remained bound after extraction with carbon tetrachloride but not after extraction with methanol was gained by determination of iodine affinity, which was greatly reduced in the carbon tetrachloride-extracted samples, but approximated that of the original amylose after methanol extraction (see Table I).

X-ray powder patterns of all complexes, as well as the amylose remaining after methanol extraction of complexes, had the pattern designated by Katz (7) as the "V" (Verkleisterung) pattern (Table II). The spacings corresponded closely to those previously reported for complexes of fatty acids with amylose (11). The minor differences between the spacings found in the various samples were probably caused by the differences in the structures of the complexing agents, differences in the amounts of the surfactants present in the complexes, and differences in the conditions under which the complexes were prepared, purified, and stored. Samec, Katz, and Derksen (20) early pointed out that slight differences between the diameters of corresponding interference rings can occur with starch preparations having the same pattern, and that apparent trifles in preparation of the materials will result in differences in the sharpness of the lines. Bear (2) has discussed some of the factors influencing the "V" pattern in detail.

The close agreement of the X-ray diagrams of the amylose-surfactant complexes with those of the fatty acid complexes indicated a similar structure, presumably the helical structure proposed for the fatty acid-amylose complexes (10). Although the surfactants with two hydrocarbon chains assume a wedge shape when present at the interface between water and another immiscible liquid, the spacings of their amylose complexes indicated that the diameter of amylose helix was only large enough to accommodate a single hydrocarbon chain. Lecithin, with its third substituent on the glycerol moiety, also formed a complex of similar dimensions, although the triglycerides of soybean oil failed even to yield a precipitate. The cause of the low degree of

TABLE I

IODINE AFFINITY OF AMYLOSE-SURFACTANT COMPLEXES

SURFACTANT	DESCRIPTION	AFTER EXTRACTION WITH CCl_4	AFTER EXTRACTION WITH CH_3OH
		%	%
Monostearins	Minimum monoglyceride content, 90%; composition, 90% stearate, 8% palmitate, 2% other fatty acid residues	1.2 3.2 5.1	17.9 17.8 18.0
Monopalmitins	Minimum monoglyceride content, 95%; composition, 95% palmitate, 5% stearate	1.6	15.7
1-Monopalmitin	90% pure	1.8	15.3
2-Monopalmitin	95% pure	2.0	14.5
D-Glucose 3-stearate	White crystals; research product[a]	0.6	13.6
D-Glucose 3-palmitate	White crystals; research product	0.8	15.3
Methyl α-D-glucoside 6-stearate	White powder; research product	0.9 0.6	14.6 18.3
Methyl α-D-glucoside 6-palmitate	White powder, m.p. 83°–86°C.; research product	1.1	14.4
Methyl α-D-glucoside 6-laurate	White powder, m.p. 68°–71°C.; research product	3.4	15.5
Sucrose monostearate	Monoester, 88%; esterification probably largely in position 6 of glucose moiety	0.6	14.5
Polyoxyethylene (8) monostearate	MYRJ 45; 37.8 – 47.2% monoester, average polymer lengths 7.25–7.50 oxyethylene units[a,b]	2.3 1.7	14.9 15.8
Oleic acid	U.S.P.	2.8	17.3
Palmitic acid		8.8	16.2
Diglycerides from hydrogenated soybean oil	Diglyceride content, 95%	5.8	12.4
Methyl α-D-glucoside distearate	Esterification predominantly on carbons 2 and 6; not homogeneous	1.3	12.3
Sucrose distearate	Diester 91.3%; esterification probably largely on carbons 6 of glucose and fructose moieties	2.3 4.3	13.0 13.8
Lecithin	Soybean phosphatides, 95+%, containing true lecithin, cephalin, and lipositol	4.8 5.5 3.3	13.6 13.5 13.4
Sorbitan tetrastearate	Technical grade	2.8	16.2

[a] See reference 13.
[b] See reference 4.

complexing by the diglycerides of hydrogenated soybean oil, indicated by the small yield of precipitate and also by their small effect on iodine affinity of amylose, is not readily apparent.

Exhaustive methanol extraction of the amylose-surfactant complexes resulted in amylose which retained the "V"-type X-ray diffraction pattern (Table II). However, the *d*-spacings were slightly smaller than those found for the corresponding complexes, although Rundle and Edwards (16) had reported the dry precipitate from the butanol complex (presumably butanol-free) to have a somewhat larger

TABLE II

X-RAY DIFFRACTION PATTERNS OF AMYLOSE-SURFACTANT COMPLEXES[a]

SURFACTANT	AFTER EXTRACTION WITH CCl_4[b]				AFTER EXTRACTION WITH CH_3OH[b]					
Monostearins	12.33m	7.06m	4.51s	3.17m	11.18m	6.99m	5.01vw	4.28s		
	12.26m	6.99m	4.48s	3.16s	11.42m	6.99m	5.08vw	4.26s	3.35vw	2.92vw
	12.00m[c]	6.97m	4.49s[c]	3.17s						
Monopalmitins	11.86s	6.95s	4.46vs	3.17m	11.13s	6.59s		4.27vs		
1-Monopalmitin	11.73m	6.93m	4.48s	3.17m						
2-Monopalmitin	11.42m	7.02m	4.48s	3.17s						
D-Glucose 3-stearate	11.79m	6.90m	4.47s	3.17m	11.24m	6.59m		4.31s		
D-Glucose 3-palmitate	11.79m	6.95m	4.49s	3.16m	11.42m	6.65m		4.31s		
Methyl α-D-glucoside 6-stearate	11.79m	7.02m	4.49s	3.16s						
	11.42m	7.02m	4.49s		11.24m	6.57m		4.29s		
Methyl α-D-glucoside 6-palmitate	12.06s	6.86s	4.49s	3.16s	11.36s	6.63s	5.02vw	4.31s		
Methyl α-D-glucoside 6-laurate	12.19s	7.02s	4.49vs	3.16m	11.48s	6.63s	5.08vw	4.27vs		
Sucrose monostearate	11.18m	6.99m	4.49s	3.16vw						
Polyoxyethylene (8) monostearate	11.79s	6.95s	4.45s	3.16s	11.54s	6.65s		4.33s		
	11.67m	6.99s	4.46vs	3.16s	11.48m	6.73s		4.35vs		
Oleic acid	11.67s	6.97s	4.44s	3.17w	11.42s	6.53s	5.07vw	4.24s	3.41w	2.91w
	12.69m	7.06m[d]	4.43m	3.16s						
Palmitic acid	11.67s	6.97s	4.48s	3.17m	11.61s	6.88s		4.31s		
Diglycerides from hydrogenated soybean oil	11.79i	7.08i	4.50s	3.16s						
Methyl α-D-glucoside distearate	11.67m	6.95s	4.46vs	3.16vw	11.27m	6.65s		4.33vs		
Sucrose distearate	11.67m	6.97m	4.49vw	3.17m						
	11.73m	6.90m	4.47s		11.36m	6.65m		4.33s	3.42vw	2.89vw
Lecithin	11.42m	7.32m	4.51s	3.17s						
	12.41s	6.95s	4.45s		11.61s	6.63s	5.09vw	4.29s		
	12.26m	6.93s	4.38s		11.24m	5.59s		4.29s	3.42vw	
Sorbitan tetrastearate	11.54m	6.97m	4.48s	3.17m						
Butanol	12.12m[e]	6.82m[e]	4.48s[e]							

[a] Intensity notation: vs, very strong; s, strong; m, medium; w, weak; vw, very weak; i, indistinct.
[b] Interplanar spacing, *d*, in angstroms.
[c] An extra diffraction line also appears at 4.09vw.
[d] An extra diffraction line also appears at 4.88m.
[e] Complex not extracted with CCl_4 before X-ray examination.

distance between helices than in either the amylose-butanol or amylose-iodine complex.

Study of the iodine uptake of amylose without isolation of the complex showed that each surfactant reduced the iodine affinity to a minimum value beyond which it could be reduced no further by addition of more surfactant, by different temperature during heating, or by longer heating time. These values are shown in Table III, together with conditions of heating which produced equilibria.

TABLE III

IODINE AFFINITY OF AMYLOSE WITH EXCESS SURFACTANT

SURFACTANT	AMOUNT ADDED[a]	CONDITIONS OF HEATING		IODINE AFFINITY
	%	*hours*	°C	%
Monostearins	54	4	84	1.7
Monopalmitins	60	3	83	1.5
1-Monopalmitin	48	4	81	2.3
2-Monopalmitin	45	4	81	1.4
D-Glucose 3-stearate	50	5	83	0.3
D-Glucose 3-palmitate	51	4	85	0.6
Methyl α-D-glucoside 6-stearate	39	4	84	0.8
Methyl α-D-glucoside 6-palmitate	41	4	83	0.9
Methyl α-D-glucoside 6-laurate	52	4	84	2.1
Sucrose monostearate	47	4	85	0.2
Polyoxyethylene (8) monostearate	37	4	81	4.5
Polyoxyethylene (40) monostearate	64	4	83	4.7
Polyoxyethlene sorbitan mono-oleate	46	4	83	4.8
Oleic acid	46	4	78	3.5
Palmitic acid	44	4	70	3.6
Sodium lauryl sulfate	43	4	84	1.5
Diglycerides from hydrogenated soybean oil	229	4.75	85	11.7
Methyl α-D-glucoside distearate	55	5	85	3.0
Sucrose distearate	46	4	70	3.3
Lecithin	56	4	84	2.1
Sorbitan tetrastearate	50	6	66	4.1

[a] Based on dry weight of amylose.

Temperatures used were influenced by the fact that some of the additives, especially the monoglycerides, became much less soluble near 100°C. The surfactants themselves were found to have no effect on the iodine titration in the absence of amylose. The iodine affinity of the amylose was reduced below 5% by all of the surfactants used except the diglycerides. The latter preparation reduced it only to 11.7%, thus exhibiting the same reluctance to complex that had been found during preparation of samples for X-ray analysis.

More striking than the differences in total iodine uptake were the differences in the slopes of the titration curves for amylose treated with excess amounts of surfactants of various structures. The compounds with one fatty acid moiety (Fig. 1) gave curves completely free

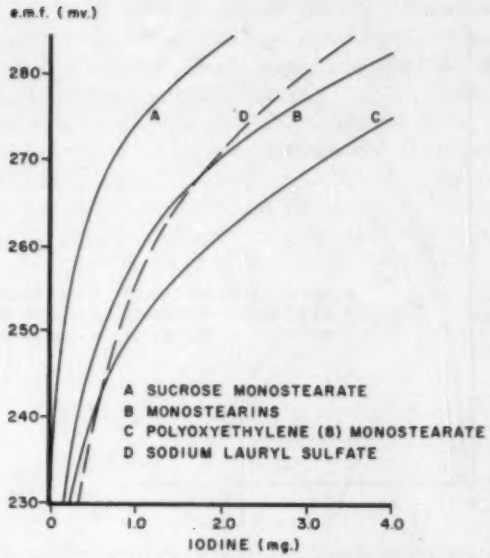

Fig. 1. Titration curves of amylose treated with excesses of some monoesters.

of the sigmoid shape characteristic for free amylose. Sucrose monostearate gave a curve which rose very rapidly; those for D-glucose 3-stearate and D-glucose 3-palmitate were very similar to it. The curves for all the monoglyceride preparations used (Table III) were almost identical, regardless of the position of the ester group in the molecule or whether the fatty acid group was palmitic or stearic, and rose somewhat less sharply than that for sucrose monostearate (Fig. 1). Polyoxyethylene (8) monostearate and the other polyoxyethylene compounds produced curves which rose still more gradually, but all three were alike.

Potentiometric titration curves of amylose to which excess palmitic or oleic acid had been added were nearly identical with that for polyoxyethylene (8) monostearate, but addition of sodium lauryl sulfate caused the curve to rise more rapidly (Fig. 1).

The esters of methyl α-D-glucoside lay between those for sucrose monostearate and the monoglycerides (Fig. 2), but were of special interest because the weight of iodine required to raise the e.m.f. to 280 mv. varied inversely with the length of the fatty acid residue.

Lecithin yielded a titration curve similar to those for the monoesters, but all the other additives examined which had more than one fatty acid moiety caused the titration curves to be sigmoid in shape

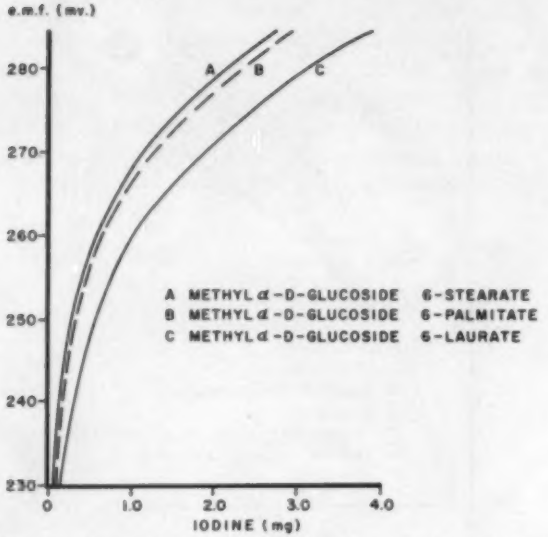

Fig. 2. Titration curves of amylose treated with excesses of methyl α-D-glucoside esters.

(Fig. 3). Sucrose distearate, methyl glucoside distearate, and sorbitan tetrastearate all caused great reductions in iodine affinity (Table III),

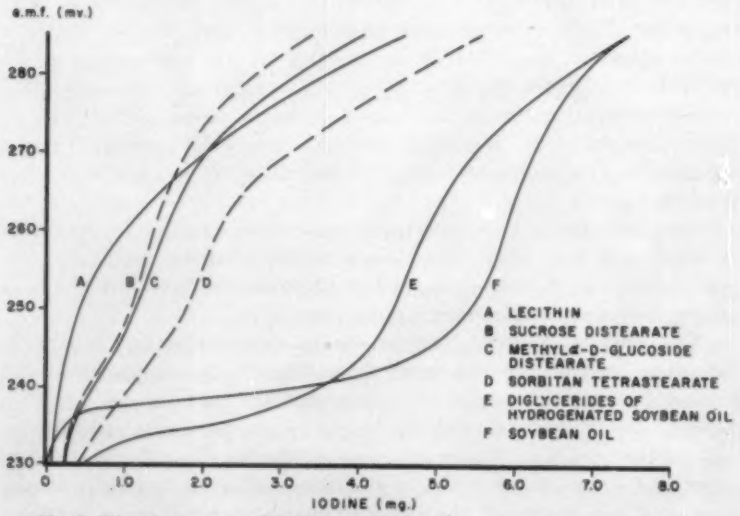

Fig. 3. Titration curves of amylose treated with excesses of some polyesters.

however. The diglyceride compound, on the other hand, was very much less effective in lowering the iodine affinity and yielded a titration curve which was much more like that produced by the addition of fat or with no additive at all.

Curves of sigmoid shape were characteristic of those titration mixtures in which little or no complex formation had occurred (i.e., with fat or diglycerides), as well as those in which the amount of additive present was less than that required to reduce iodine affinity to its minimum value (Fig. 4). It therefore seems probable that the sigmoid

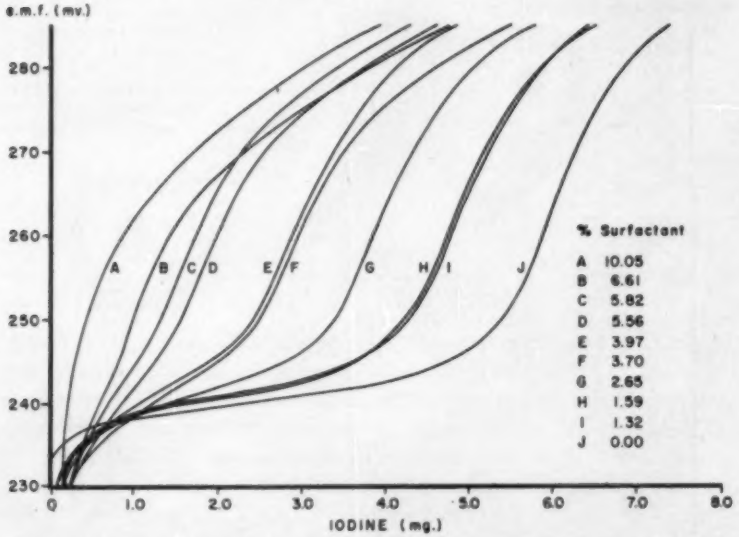

Fig. 4. Titration curves of amylose treated with different amounts of 1-palmitin.

shapes of the curves for mixtures containing sucrose distearate, methyl glucoside distearate, and sorbitan tetrastearate were evidence of the inability of these compounds to complex the amylose completely. Although the mixtures containing large amounts of these surfactants had iodine affinities no higher than those with many of the other surfactants used, they also differed in that addition of a small amount of iodine at the start of the titration immediately produced the blue color typical of the starch-iodine complex.

The slopes of the curves obtained with the surfactants containing only one ester group may be related to the stabilities of the complexes formed, since those with the greatest slopes (Fig. 1) show the greatest reduction in iodine affinity (Table III). The smaller slopes show less

reduction in iodine affinity, possibly because iodine may have replaced some of the surfactant.

Titration curves gradually lost their pronounced sigmoid shape as the amount of surfactant added approached that required for complete complex formation (Fig. 4). Similar series of curves had been obtained by Mikus, Hixon, and Rundle (10) when increasing amounts of oleic and palmitic acids were added to amylose. A linear relationship was observed between the percentage of surfactant added and the resulting iodine affinity (Fig. 5), until the iodine uptake was reduced to nearly

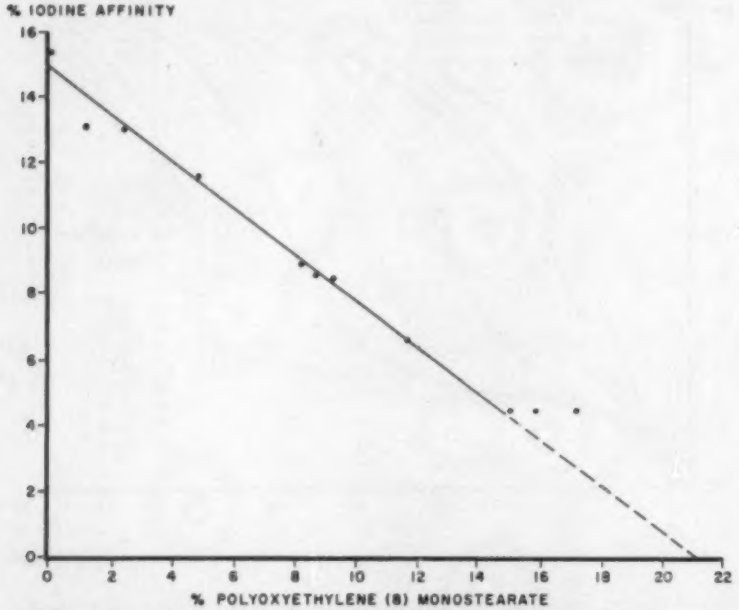

Fig. 5. Relationship between percentage of polyoxyethylene (8) monostearate added and iodine affinity of amylose.

the maximum extent which had been determined previously by use of large excesses of the surfactant. Although none of the surfactants reduced the iodine affinity to zero, the linear portion of the curves could be extrapolated to intercept the X-axis and provide values for comparison of the various surfactants with each other and with values reported by other investigators (9,21,23). The values so obtained represent the percentage of each surfactant which would be needed to reduce the iodine affinity of the amylose to zero if the iodine affinity did not reach a limiting minimum value, as already noted. They varied

from 5.4 to 29.8% of the weight of the amylose and were higher for compounds of greater molecular weight (Table IV). For surfactants

TABLE IV

WEIGHT OF SURFACTANT COMPLEXED COMPARED TO ITS STRUCTURE

SURFACTANT	NEEDED FOR ZERO IODINE AFFINITY [a]	MOLECULAR WEIGHT	HYDROCARBON CHAIN IN MOLECULE
	%		%
Palmitic acid	5.4	256	82
Monostearins	6.4	359	67
Monopalmitins	7.5	331	64
Methyl α-D-glucoside 6-palmitate	8.4	433	49
Sodium lauryl sulfate	9.3	288	59
D-Glucose 3-stearate	10.0	447	54
Methyl α-D-glucoside 6-stearate	10.0	461	52
Methyl α-D-glucoside 6-laurate	11.3	376	41
D-Glucose 3-palmitate	15.4	419	50
Polyoxyethylene (8) monostearate	21.0 (17.6)[b]	679[c]	43 (34)[d]
Lecithin	29.8	790[e]	60 (30)[d]

[a] Calculated by extrapolation of linear portion of curve (cf. Fig. 5); actually iodine affinity reached minimum value (Table III) below which no further reduction could be obtained.
[b] Estimated weight of esters in sample, without free polyglycols (4).
[c] Estimated molecular weight of mono- and di-esters in sample (4).
[d] Estimated percentage of molecule composed of a single hydrocarbon chain (assuming the additional hydrocarbon chains in molecule are unable to form complex).
[e] Calculated for lecithin molecule containing two stearoyl groups.

with a single hydrocarbon chain, the amount of surfactant required appeared to be still more closely related to the percentage of the molecule composed of the hydrocarbon chain, although the amounts of D-glucose 3-palmitate and polyoxyethylene (8) monostearate needed were appreciably higher than would be anticipated from such a relationship. The former, however, has previously been shown to give results different from those which might be expected in its action on both crumb firmness (10) and Brabender Amylograph curves of starch pastes (12).

The action of the polyoxyethylene (8) monostearate preparation in reducing iodine affinity may have been lessened considerably by the presence of a large proportion of the diester (4). As mentioned above, diglycerides are very inefficient in reducing iodine affinity, and lecithin, although much more effective, is far less so than the majority of monoesters studied.

Effects of surfactants on crumb firmness in bread (11) and on the Brabender Amylograph curves of corn starch pastes (12) do not appear to be directly related to their complex formation with amylose under the conditions of these experiments or to the cell dimensions of the complexes. However, the possibility remains that they may differ in their abilities to form complexes under the conditions present in starch pastes or in bread.

Acknowledgments

The authors wish to express their appreciation to G. L. Clark of the Department of Chemistry for his counsel and the use of equipment in making the X-ray analyses, and to Paul E. McMahan for determining the infrared absorption spectra. They also wish to thank the Corn Industries Research Foundation for a grant for support of this study and the following donors of materials used: Distillation Products Industries, Procter and Gamble Company, Corn Products Company, A. E. Staley Manufacturing Company, Herstein Laboratories, and the Northern Utilization Research and Development Branch of the Agricultural Research Service, U.S. Department of Agriculture.

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THE OXIDATION OF WHEAT FLOUR
III. The Isolation of Thioctic Acid[1]

BETTY SULLIVAN, LELAND K. DAHLE, AND DAVID A. PETERSON

ABSTRACT

Thioctic acid, a coenzyme, was isolated from wheat flour. The compound was identified by its sulfur content, by the melting point of its S-benzyl thiuronium derivative, by its R_f value, and by its ultraviolet spectrum.

It is estimated that the compound is present in amounts of approximately 1 to 10 p.p.m., although less than this amount was recovered with the isolation procedure used. Wheat germ, as might be expected, contains a much greater amount of thioctic acid than flour.

In a recent paper, Dahle and Sullivan (2) reported the presence of thioctic acid in the acid-hydrolyzed extract of wheat flour. The compound was identified by the absorption maximum at 334 mμ and by chromatography. This paper will describe the isolation and further identification of thioctic acid from wheat flour.

Materials and Methods

A straight-grade, unbleached, spring wheat flour of 0.43% ash and 13.0% protein was used as the source material. All chemicals were analytical reagent grades.

The isolation procedure was as follows: A total of 8.9 kg. of flour was hydrolyzed in eight batches of 1,000 g. each and one batch of 900 g. Two liters of water and 65 ml. of concentrated hydrochloric acid were mixed with 1,000 g. of flour and autoclaved for 1 hour at 126°C. The suspension was cooled and centrifuged at 1,700 r.p.m. for 25 min-

[1]Manuscript received March 30, 1961. Contribution from the Research Laboratories of Russell Miller-King Midas Mills, Minneapolis, Minnesota.

utes. The water-soluble layer was extracted with benzene, using a ratio of one part benzene to three parts of the water extract. The extract was washed twice with small amounts of benzene. During the extraction with benzene, emulsions formed occasionally; they were broken by centrifugation. The benzene extract was then extracted with 5% sodium bicarbonate, using about one part of sodium bicarbonate solution to four parts of the benzene extract. The water extract, containing the sodium salts of any acids present, was acidified with 6*N* hydrochloric acid until the pH was less than 1.0. The solution changed from a brown to a yellow color and was allowed to stand for 15 to 30 minutes until the carbon dioxide escaped. The combined acid solutions from all batches (2 parts) were then extracted with ethyl ether (1 part) and the ether solution evaporated to about 2 ml. This solution was introduced into a silica-powder-1% ether column about 4 cm. in length and 1.5 cm. in diameter. The ether solution and subsequent eluting solutions were forced through the column with slight air pressure. Fatty acids were eluted with three washings of 1% ethyl ether in petroleum ether—first 4 ml., then 2 ml. and, finally, 1 ml. Thioctic acid and a quinone-type pigment were eluted with two washings of ethyl ether and concentrated to about 1 ml. The crude thioctic acid was esterified by refluxing 1 hour with 75 ml. of ethanol and a drop of hydrochloric acid. After cooling, the ethanol solution was diluted with twice its volume of water and extracted with ethyl ether. The ether solution was washed with cold 5% sodium bicarbonate to remove the pigment, then with 0.1*N* hydrochloric acid and, finally, with water. The ester was then hydrolyzed to the acid by gentle refluxing with 0.1*N* hydrochloric acid and the acid extracted with chloroform and, finally, recrystallized from petroleum ether. A few milligrams of yellow crystals were isolated.

Identification of Thioctic Acid

The following means were used to identify the compound as thioctic acid.

1. *Ascending Strip Chromatography.* A benzene extract was applied by micropipet in two or three drops of 10 to 15 μl. each to a strip of Whatman No. 1 filter paper (17 cm. by 3 cm.) using 1% glacial acetic acid as the solvent. After 1 hour, the paper was immersed in 0.1*N* potassium permanganate, withdrawn, and, after 1 minute, rinsed in a large quantity of distilled water. A brown spot having an R_f value of 0.70 was found. The same value was obtained using pure dl-thioctic acid.

2. *Ultraviolet Spectrum.* The yellow extract in ether gave a maximum absorbance at 334 mμ. Synthetic thioctic acid (1) and the isolated

compound gave identical absorbance maxima.

3. *S-Benzyl Thiuronium Derivative.* This derivative was made according to the procedure of Donleavy (3). Ether was evaporated from the solution of thioctic acid isolated from flour by the above procedure. Sodium bicarbonate (2 mg.), water (10 ml.), and 1 drop of 1.0*N* hydrochloric acid were added. This solution was added to a boiling solution of 7.2 mg. of S-benzyl thiuronium chloride in 10 to 15 ml. of ethanol. After evaporation to 1 to 2 ml. and cooling in an ice bath, crystals were obtained showing a melting point of 140°C. The derivative made in a similar manner from pure dl-thioctic acid and recrystallized from absolute methanol gave a melting point of 140°C. The only reference in the literature is that of Segre, Viterbo, and Parisi (4), who reported a melting point of 152° to 154° of the S-benzyl thiuronium derivative crystallized from absolute alcohol and another value of 132° to 134° observed once. They indicated that this wide discrepancy might be due to crystallization and drying.

4. *Analysis of Thioctic Acid.* The material isolated from flour was found to contain 31.87% sulfur. The theoretical sulfur content of thioctic acid, $C_8H_{14}O_2S_2$, is 31.1%. Since less than 1 mg. was used for analysis, the agreement between the theoretical and the sulfur as found was considered adequate.

Wheat germ, as might be expected, contains much more thioctic acid than flour; our present estimate is 40 to 50 times the amount in flour.

Discussion

Thioctic acid is a coenzyme generally considered to be bound to a protein; hence there is need of an acid hydrolysis to release it. Recent work in the development of a method for the measurement of thioctic acid indicates that the acid hydrolysis conditions employed may have caused significant losses. Present methods would indicate that flour contains from 1 to 10 p.p.m. of thioctic acid and wheat germ about 200 to 300 p.p.m.

Further work is needed on the best conditions for hydrolysis and reduction before accurate figures can be given.

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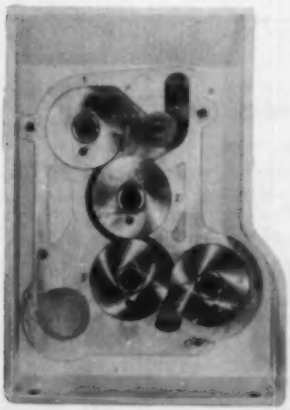

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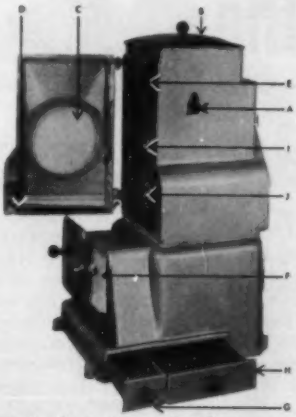

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1435 Clay St., P. O. Box 7474
Kansas City 16, Mo.

• Tennant & Hoyt, Lake City, Minnesota. Pneumatic mill producing 5500 sacks per day. Elevator storage 600,000 bu.

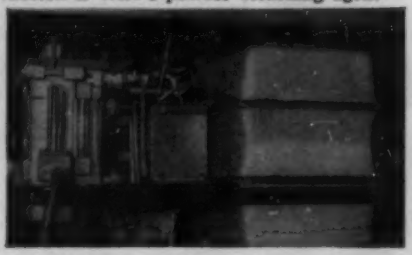
TENNANT & HOYT
KEEPS OVEN SPRING WORKING IN SPRING WHEAT FLOURS
with W&T FLOUR TREATMENT

To give its strong spring wheat flours that extra kick, Tennant & Hoyt chooses W&T flour treatment.

Experience proves that Dyox® strengthens gluten to a maximum, makes doughs more machinable, prevents "stick-ups". And there's no waste with W&T's Dyox. Accurate proportioning equipment makes chlorine dioxide gas as it is needed—applies it uniformly in precise amounts.

Along with Dyox, Tennant & Hoyt uses Novadelox® to whiten and brighten flour . . . give it the color consumers prefer. Novadelox is W&T's powder bleaching agent—made by W&T, distributed by W&T.

Tennant & Hoyt is only one of the many milling companies using W&T flour treatment. If your mill is not one of these, investigate the advantages of Wallace & Tiernan's complete flour service.



Proportioning assembly of Dyox machine. This is the heart of the Dyox Process. Here, proportioned amounts of air, chlorine, sodium chlorite are metered. When mixed in a reaction tower, chlorine dioxide gas results. This is generated as needed and metered as a gas not a liquid.

WALLACE & TIERNAN INC.

NOVADEL FLOUR SERVICE DIVISION
25 MAIN STREET, BELLEVILLE 9, NEW JERSEY

N-121.20